Measurement of HLA class I expression in ankylosing spondylitis

Paul Creamer, John Edmonds, John Sullivan, Sita Matthews

Abstract

The importance of HLA-B27 in the pathogenesis of ankylosing spondylitis is uncertain: current evidence favours a role for the B27 molecule itself. The possibility that quantitative differences in HLA-B27 expression may exist between patients with ankylosing spondylitis, family members, and control subjects positive for B27 was examined using appropriate monoclonal antibodies, flow cytometry, and a 'model lymphocyte' coated with a known number of mouse immunoglobulin binding sites. No differences were found between the groups. HLA-A2, examined for comparison, was expressed in greater amounts than HLA-B27, but each contributed only 10-20% of the total class I antigens. Heterozygotes expressed twice the amount of antigen expressed by homozygotes. Synovial lymphocytes expressed more class I antigens than peripheral lymphocytes.


Although many human diseases are associated with particular major histocompatibility complex (MHC) groups\(^1\) the association between ankylosing spondylitis and HLA-B27 is one of the strongest known, with over 90% of affected subjects being HLA-B27 positive.\(^2\)\(^,\)\(^3\) It is also one of the few examples of a class I associated disease. Despite the strength of the association the part played by B27 in the pathogenesis of ankylosing spondylitis remains unclear. The gene linkage theory, which suggests that HLA-B27 is simply a marker for another closely linked ankylosing spondylitis gene, has little evidence to support it. Genetic studies\(^4\) and analysis of restriction fragment length polymorphisms\(^5\) have not found a distinct ankylosing spondylitis susceptibility gene. It seems increasingly likely that ankylosing spondylitis is the result of an immune response targeted to the B27 molecule itself.\(^6\) The nature of this response is as yet unclear; the two major theories which have been proposed require either molecular mimicry\(^7\) or expression of a novel B27 associated structure in subjects with ankylosing spondylitis.\(^8\) Clearly the two theories presuppose expression of the B27 antigen on relevant target tissues.

Although class I antigens are widely expressed\(^9\) they are by no means universal. Enhanced expression in response to some cytokines has been shown in vitro\(^10\) and in vivo\(^11\)\(^,\)\(^12\) and tissues that do not normally express class I antigens may be induced to do so by the same cytokines.\(^13\) Little is known about quantitative differences in expression between various sites or under various conditions. Moreover, we do not know if the expression of, say, HLA-B27 differs from that of other class I molecules.

Quantitative differences in soluble mediators of inflammation are thought to be important in the pathology of several rheumatic diseases. Various cytokines, for example, are known to be increased or depressed in rheumatoid arthritis.\(^14\) The effects of differential expression of other components of the immune system are not clear. The MHC plays an integral part by presenting processed foreign antigen to T cells\(^15\) and it is therefore at least possible that variations in the levels of MHC expression may exert an influence on the immune response.

We have set out to examine differences in total class I and HLA-B27 expression in subjects with ankylosing spondylitis, their family members, and HLA-B27 positive healthy controls using appropriate monoclonal antibodies and flow cytometric analysis. Relative differences were sought using mean fluorescent channel as a measure of bound antibody. An attempt was made to derive actual numbers of class I molecules using a standard 'model lymphocyte' with a known number of mouse immunoglobulin binding sites (Simply Cellular Microbeads, Flow Cytometry Standards Corporation, Research Triangle Park, NC, USA).

Subjects and methods

All 11 patients with ankylosing spondylitis were diagnosed according to the New York criteria.\(^16\) The 10 family members were siblings, children, or parents of affected subjects. The seven HLA-B27 positive controls were healthy blood donors. All subjects were typed for HLA-A and B alleles. All were B27 positive. Subjects with HLA-B27 were excluded owing to possible cross-reactivity with the monoclonal antibody to HLA-B27.\(^17\) Three subjects (two with ankylosing spondylitis, one control) were homozygous for HLA-B27 on serological testing. Absence of disease in family members and controls was confirmed by a questionnaire and examination of the spine. For subjects with ankylosing spondylitis the erythrocyte sedimentation rate (ESR) was measured as an index of disease activity. Other than HLA typing no blood tests were performed on healthy subjects.

Synovial lymphocytes were obtained from three subjects with various rheumatological diseases for simultaneous comparison with peripheral lymphocytes (see table 5).
Peripheral blood was collected into heparinised tubes. Mononuclear cells were separated by Ficoll-Hypaque density centrifugation and stored in the vapour phase of liquid nitrogen in the presence of 10% dimethylsulphoxide until analysed. Synovial fluid lymphocytes were prepared according to the method of Ford et al. Viability was checked by trypan blue exclusion and was always greater than 85%.

**REAGENTS**

Primary antibodies used were monoclonal antibodies directed against total HLA class I, \( \beta_2 \)-microglobulin, HLA-B27, and HLA-A2 (table 1). The fluorescein conjugated second step reagent for indirect immunofluorescence was a goat antimouse immunoglobulin (Silenus Laboratories, Australia).

**IMMUNOFLUORESCENT STAINING**

Briefly, \( 2 \times 10^6 \) mononuclear cells were incubated with 100 \( \mu l \) of primary antibody, followed by secondary incubation with 100 \( \mu l \) of fluoresceinated second step reagent. Incubations were for 60 minutes at 4°C. Washes between and after incubations were with 1% bovine serum albumin-phosphate buffered saline (BSA-PBS) (pH 7.4). Cells were fixed in 0.5% paraformaldehyde and stored in the dark at 4°C until analysed (within 48 hours).

Primary antibody was omitted from negative controls. All antibodies were used at saturating concentrations determined by titration of the antibody against the mean fluorescent channel. All samples were analysed in duplicate and the mean was used for calculations.

**FLOW CYTOMETRY**

Fluorescent staining was evaluated using a FacScan (Becton Dickinson, Mountain View, CA, USA) and FacScan Research software. The machine uses a 15 mW 488 nm air cooled argon ion laser. After an initial test period standard operating settings were determined for fluorescence signal processing. These standard operating conditions were maintained for all test data. Raw data were analysed using a preset keystroke programme to facilitate uniform data interpretation.

**MEASUREMENT OF ANTIGEN**

The number of fluorescein isothiocyanate (FITC) molecules bound to each cell was calculated from the mean fluorescent intensity using a standard calibration graph determined from flow cytometric measurements of standard beads expressing \( 7 \times 10^{-3} \)-1.8 \( \times 10^6 \) FITC molecules per bead (High and Low Level Quantitative Fluorescent Microbead Standards, Flow Cytometry Standards Corporation) (fig 1). The FITC to protein (F/P) ratio was determined for each monoclonal antibody using Simply Cellular Microbeads (Flow Cytometry Standards Corporation). These are designed to be used as ‘model lymphocytes’ and are coated with a fixed number of goat antimouse \( \beta_2 \)-microglobulin binding sites (for the batch used in these studies, \( n=1 \times 10^5 \)). They react uniformly with murine monoclonal antibodies of IgG1, IgG2a, and IgG2b isotypes. The F/P ratio was calculated by dividing the number of FITC molecules bound to the bead by the number of binding sites on the bead (1.1 \times 10^5), the monoclonal antibody being used at saturating concentrations. The mean number of binding sites on each cell was calculated by dividing the difference in the number of FITC molecules for each cell and the corresponding negative control by the F/P ratio for that antibody. This example calculation is illustrated in fig 1. In this experiment, using monoclonal antibody PA2.6, the mean fluorescent channel for the beads was 673, corresponding to 1.5 \times 10^5 FITC molecules. The mean fluorescent channel for the control (not illustrated) was 178, corresponding to 0.015 \times 10^5 FITC molecules. The F/P ratio was therefore 1.5 \times 10^5-0.015 \times 10^5 divided by 1.1 \times 10^5, that is, 1.35. The mean fluorescent channel for cells was 820, corresponding to 5.0 \times 10^5 FITC molecules. The control value for cells was 0.016 \times 10^5. The number of binding sites on each cell was therefore 5.0 \times 10^5-0.016 \times 10^5 divided by 1.35, that is 3.69 \times 10^5.

Incubations for microbeads were performed at room temperature with a washing solution of 0.05% Tween/1% BSA-PBS (pH 7.4) and the beads were not fixed before analysis. Otherwise the beads were treated in the same way as cells. Saturating concentrations were used throughout. All samples using microbeads were analysed in triplicate.

**Table 1** Monoclonal antibodies used in indirect immunofluorescence

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Ig or CS*</th>
<th>Immunoglobulin class</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA ABC.m3†</td>
<td>Ig</td>
<td>IgG2x</td>
<td>HLA-B2722</td>
</tr>
<tr>
<td>BB7.2†</td>
<td>CS</td>
<td>IgG2x</td>
<td>HLA-A2b</td>
</tr>
<tr>
<td>BBM †</td>
<td>CS</td>
<td>IgG2b</td>
<td>Human ( \beta_2 )-microglobulin23</td>
</tr>
<tr>
<td>PA2.6‡</td>
<td>CS</td>
<td>IgG1</td>
<td>MHC class I glycoprotein24</td>
</tr>
</tbody>
</table>

* Ig=isolated immunoglobulin; CS=culture supernatant.
† Silenus Laboratories, Victoria 3122, Australia.
‡ American Type Culture Collection, Rockville, MD 20852, USA.
Table 2: Characteristics of groups and mean fluorescence channel values (mean for group)

<table>
<thead>
<tr>
<th>Group</th>
<th>No of subjects (no of men)</th>
<th>Mean age (range)</th>
<th>Mean fluorescence channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankylosing spondylitis</td>
<td>11(10)</td>
<td>42 (26-55)</td>
<td>Control: 214, 820, 637, 722</td>
</tr>
<tr>
<td>Family</td>
<td>10(5)</td>
<td>34 (16-66)</td>
<td>B27 positive (heterozygote): 214, 820, 637, 722</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>7(5)</td>
<td>B27 positive (homozygote): 214, 820, 634, 722</td>
</tr>
</tbody>
</table>

Table 3: Measurement of binding sites: comparison of heterozygotes with homozygotes for HLA-B27 and HLA-A2. Results are mean (SD) binding sites (×10^5/cell).

<table>
<thead>
<tr>
<th>Group</th>
<th>Class I</th>
<th>B27</th>
<th>%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B27 heterozygotes (n=25)</td>
<td>4.34 (1.54)</td>
<td>0.49 (0.09)</td>
<td>11.6 (3.49)</td>
</tr>
<tr>
<td>B27 homozygotes (n=3)</td>
<td>3.11 (0.68)</td>
<td>0.01 (0.01)</td>
<td>32.7 (6.75)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Class I</th>
<th>A2</th>
<th>%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2 heterozygotes (n=5)</td>
<td>3.11 (0.48)</td>
<td>0.62 (0.11)</td>
<td>20.5 (5.00)</td>
</tr>
<tr>
<td>A2 homozygotes (n=5)</td>
<td>3.15 (0.49)</td>
<td>1.25 (0.13)</td>
<td>40.3 (5.11)</td>
</tr>
</tbody>
</table>

%* = B27 or A2 as percentage total class I.

Table 4: Correlation of PA 2.6 and BBM.1 antibodies (all values = binding sites ×10^5/cell).

<table>
<thead>
<tr>
<th>Subject No</th>
<th>Class I (PA2.6)</th>
<th>B2 Microglobulin (BBM.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.37</td>
<td>6.67</td>
</tr>
<tr>
<td>2</td>
<td>4.83</td>
<td>5.80</td>
</tr>
<tr>
<td>3</td>
<td>5.30</td>
<td>7.45</td>
</tr>
<tr>
<td>4</td>
<td>5.58</td>
<td>6.87</td>
</tr>
<tr>
<td>5</td>
<td>5.68</td>
<td>8.71</td>
</tr>
<tr>
<td>6</td>
<td>5.77</td>
<td>8.52</td>
</tr>
<tr>
<td>7</td>
<td>6.70</td>
<td>9.10</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>5.46(0.74)</td>
<td>7.59(1.22)</td>
</tr>
</tbody>
</table>

Correlation coefficient = 0.82 (p<0.05).

Results

Table 2 gives the characteristics of the studied groups. Mean disease duration in the group with ankylosing spondylitis was 18.9 years (range 4-32 years). The mean ESR of the group with ankylosing spondylitis was 26 mm/hour (range 7-61). No correlation was seen between the ESR and class I expression (data not shown). Overall the mean fluorescence channel for the negative control (FITC labelled whole) was 183 (mean) and was never higher than 227. This corresponded to a maximum of 3×10^5 FITC molecules per cell, or less than 1% of the value obtained for class I antigens. Mean fluorescence channel values were similar for all groups for total class I and HLA-B27.

Measurement of binding sites (fig 2) showed a similar amount of class I antigen in all three groups. HLA-B27, though also constant, appeared to be contributed only 10-12% of total class I antigen in homozygotes. Again, no differences were found between the groups.

Homozygotes expressed more HLA-B27 than heterozygotes as evidenced by higher mean fluorescence channel values. Measurement showed the number of sites to be almost exactly double in homozygotes (1.01×10^5×0.49×10^5) (table 3).

Levels of HLA-A2 in five heterozygote subjects (one with ankylosing spondylitis, one family and three controls) were higher than HLA-B27 at 0.62×10^5/cell, compared with 0.49×10^5/cell for HLA-B27. Homozygotes (three with ankylosing spondylitis, two family) again expressed double the amount of heterozygotes (table 3).

BBM.1, an antibody directed against β2 microglobulin, was examined in seven subjects (two with ankylosing spondylitis, five controls). It detected a larger number of binding sites than antibody PA2.6 (7.59×10^5×5.46×10^5 sites on each cell) but the two were significantly correlated (p<0.05) (table 4).

Synovial lymphocytes invariably expressed more HLA class I antigen than simultaneously obtained peripheral blood lymphocytes (table 5). In the single subject with ankylosing spondylitis studied here the relative amount of HLA-B27 was also greater.

REPRODUCIBILITY

Intra-experiment reliability for the cells and microbeads was good, with a coefficient of variation of 2-3%. Cells from five subjects were analysed on two occasions to assess inter-experiment reliability, giving a coefficient of variation of 12-9%.

Discussion

Subjects with ankylosing spondylitis, family members, and HLA-B27 positive controls express approximately the same amount of total HLA class I antigen as evidenced by similar mean fluorescence channel values for a monoclonal antibody directed against a common determinant of class I antigen. HLA-B27 levels...
are also similar. This confirms the findings of Van der Gaag et al.\textsuperscript{24} who, using a quantitative complement mediated cytotoxicity assay, found that although the expression of HLA-B27 varied between subjects there was no significant difference between the mean titration curves for patients with ankylosing spondylitis and healthy HLA-B27 positive controls.

Measurement of these antigens by reference to a known standard gives values for HLA class I expression of the same order of magnitude as that obtained by different methods. Trucco et al.\textsuperscript{25} using radiolabelled antibodies, found levels of total class I of 1.0×10\textsuperscript{5}–2.6×10\textsuperscript{7} on peripheral lymphocytes, and B cells expressed more than T cells. Our results were higher at about 4×10\textsuperscript{7}. This probably represents methodological differences in the method used.

Our work suggests that HLA-A and HLA-B alleles are not necessarily expressed in equal amounts: we found that HLA-A2 was expressed quantitatively more than HLA-B27. The two, however, contribute only around 10–20% of total class I antigens, adding up to about 60–80% for HLA-A and HLA-B alleles. This was confirmed by analysis of a subject homozygous for HLA-B27 and HLA-A2. Binding sites for these two alleles respectively were 1.01×10\textsuperscript{5} and 1.44×10\textsuperscript{5}, totalling 2.45×10\textsuperscript{5}. The value for total class I was 3.11×10\textsuperscript{5}, leaving about 21% to be accounted for by non-HLA-A and HLA-B antigens. The implication is that a large proportion of HLA exists which is recognised by a monoclonal antibody against a common determinant of HLA A, B, and C, but which is not detected by antibodies against HLA-A and HLA-B specificities. This has been confirmed on lymphoblastoid cell lines\textsuperscript{26,27} and on soluble HLA molecules in serum, spleen, and lymphocyte cultures.\textsuperscript{28} Indeed, the first ‘non-classical’ class I antigen (HLA-E) has now gained approval from the HLA nomenclature committee.\textsuperscript{29} The distribution, expression, and function of these ‘non-classical’ antigens remains to be established.\textsuperscript{30}

Our finding that homozygotes express almost exactly double the amount of HLA as heterozygotes may have been predictable on theoretical grounds. Nevertheless, the use of this relatively simple technique may provide a useful alternative to detailed family studies as a means of confirming serological homozygosity.

β\textsubscript{2} Microglobulin was present in greater amounts than class I antigens, though levels were highly correlated (\textit{p}<0.05) in a small sample size. This is in accordance with earlier reports using this antibody.\textsuperscript{25,31} It seems likely that some β\textsubscript{2} microglobulin molecules are associated with human leucocyte antigens other than HLA heavy chains, or exist in isolation on the cell surface.

The technique of measurement using Simply Cellular Microbeads makes several assumptions, the most questionable of which is that binding of antibody to the surface of the bead occurs in the same way as to the surface of the cell. Clearly, the cell surface is a complex structure composed of hundreds of different molecules, each carrying many antigenic determinants. The possibility that one of these influences the binding of monoclonal antibodies to an adjacent HLA molecule must be considerable. Moreover, as the bead binds all mouse immunoglobulin, this is likely to be to the Fc part of the immunoglobulin, whereas binding to the cell surface will be via the F(ab)\textsubscript{2} fragment.

We experienced technical problems in achieving saturation of beads. For the cells a point was clearly reached beyond which addition of further antibody did not lead to increased mean fluorescence channel. Although considerable ‘flattening off’ of the curve was found with the beads, we were unable to show clearly a point of saturation. We conclude that non-specific binding of the antibody occurred in bead concentrations to the surface of the bead. For our purposes we estimated saturation to occur at a point beyond which doubling of the antibody concentration resulted in a less than 1% increase in mean fluorescence channel (see fig 3).

It should be noted, however, that the main alternative method of measurement (use of radiolabelled antibody) is also subject to potential errors—for example, chemical damage of reagents during iodination, or the appearance of radioactive iodine instead of tyrosine in the antigen combining sites.\textsuperscript{25} How does this information assist our understanding of the pathogenesis of ankylosing spondylitis? We found no evidence that a simple quantitative difference in HLA-B27 exists to account for disease. The possibility remains that such differences exist in an earlier phase of the disease, perhaps even before it is clinically apparent, or that cell types more clearly associated with pathological changes in ankylosing spondylitis do show differences. In the latter context it is of interest that synovial lymphocytes express demonstrably more class I antigens than simultaneously obtained peripheral lymphocytes. In the single subject with ankylosing spondylitis studied here B27 appeared to be upregulated more than other class I alleles. This is in accordance with in vitro evidence that differential upregulation of class I alleles may occur in human thymocyte cell lines\textsuperscript{32} and peripheral blood lymphocytes.\textsuperscript{33}

Conclusions
Peripheral blood lymphocytes from patients with ankylosing spondylitis do not appear to
differ quantitatively in the expression of total HLA class I or HLA-B27 from family members or HLA-B27 positive controls. Differences may exist in other cell types or at different stages of the disease. HLA-A and HLA-B contribute only about 80% of binding sites obtained with an antibody directed against total 'classical' HLA class I.

Use of a 'model lymphocyte' coated with a known number of antibody binding sites allows relatively simple determination of cell surface markers. Although certain assumptions are made in their use, we found that for a study of HLA class I expression results were consistent with conventionally obtained values and compatible with our current knowledge in this field.

Paul Cremer was a recipient of a Boots/Arthritis and Rheumatism Council Travelling Fellowship. We thank Lynsey Peters for assistance with flow cytometric analysis, also Margaret Cooley. Sara Browning provided valuable secretarial help.