Two HLA-B14 Subtypes (B*1402 and B*1403) Differentially Associated with Ankylosing Spondylitis Differ Substantially in Peptide Specificity but Have Limited Peptide and T-cell Epitope Sharing with HLA-B27*

Elena Merino, Verónica Montserrat, Alberto Paradela, and José A. López de Castro

From the Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, Facultad de Ciencias, Universidad Autónoma, 28049 Madrid, Spain

The peptide specificity of HLA-B*1403, an allotype associated with ankylosing spondylitis (Lopez-Larrea, C., Mijiyawa, M., Gonzalez, S., Fernandez-Morera, J. L., Blanco-Gelaz, M. A., Martinez-Borra, J., and Lopez-Vazquez, A. (2002) Arthritis Rheum. 46, 2968–2971) was compared with those of the non-associated B*1402 and the prototypic disease-associated B*2705 allotypes. Although differing by a single residue (L156R), B*1402 and B*1403 shared only 32–35% of their peptide repertoires. Subtype-related differences observed in multiple peptide positions, including P3 and P7, were largely explained by a direct effect of the L156R change on peptide specificity. The HLA-B14 subtypes shared only ~3% of their peptide repertoires with B*2705. This was due to distinct residue usage at most positions, as revealed by statistical comparison of B*1402, B*1403, and B*2705-bound nonamers. Nevertheless, shared ligands between B*2705 and B*1403 were formally identified, although ligands common to both B*2705 and B*1403, but absent from B*1402, were not found. Alloreactive T-cells were used as a tool to analyze epitope sharing among B*1402, B*1403, and B*2705. The percentage of cross-reactive T-cell clones closely paralleled peptide overlap, suggesting that shared ligands tend to maintain their antigenic features when bound to the different allotypes. Our results indicate that B*1403 and B*2705 can present common peptides. However, both the disparity of their peptide repertoires and the lack of binding features shared by these two allotypes, but not B*1402, argue against, although do not exclude, a mechanism of spondyloarthritis mediated by specific ligands of B*2705 and B*1403.

The association of HLA-B*1403 with spondyloarthropathies and, in particular, ankylosing spondylitis (AS) is, by far, the strongest one between an HLA class I molecule and any disease (1, 2). The property of HLA-B27 that determines disease susceptibility remains unknown, and several pathogenic mechanisms are currently being considered as a basis for various research lines (3, 4). One of these mechanisms is proposed by the arthritogenic peptide hypothesis (5), which assumes that an initial pathogenetic event would be a cytotoxic T lymphocyte (CTL) response against an external antigen, whose molecular mimicry with a constitutive peptide ligand of HLA-B27 would elicit an autoimmune reaction against this molecule, leading to tissue damage and inflammation. This hypothesis, which emphasizes the antigen-presenting specificity of HLA-B27 as a critical pathogenetic feature, has stimulated studies on the nature of HLA-B27-bound peptide repertoires and their relationship to those presented by other class I molecules. A major feature of HLA-B27 ligands is the presence of R2 (6, 7), which binds tightly into the pocket of the peptide-binding site (8, 9). Because this pocket is made up of residues that are polymorphic among HLA class I molecules, few allotypes other than HLA-B27 bind peptides with R2. For this reason, it was of interest that B*3901, an allotype that also accepts peptides with this motif (10) and binds in vitro some HLA-B27 ligands (11), was significantly increased among HLA-B27-negative AS patients in a study carried out in Japan (12). In Caucasian patients, HLA-B39 was associated with the axial type of psoriatic arthritis (13), a form of this disease that is strongly linked to HLA-B27 (14). In addition, development of spondyloarthopathies in gorillas has been correlated with these animals expressing MHC class I molecules that bind peptides with R2 (15).

Recently, B*1403, an allotype reported only in the African populations of Cameroon and Togo (16, 17), was found strikingly associated to AS in the Togolese population, where both this disease and HLA-B27 are rare (17). Of 7 HLA-B27-negative AS patients that could be clinically and genetically evaluated, 4 (57%) were of the B*1403 allotype. The prevalence of this allotype in the normal Togolese population was <1%. The closely related allotype B*1402, which differs from B*1403 only at residue 156 (Leu in B*1402; Arg in B*1403), was found in 2% of the healthy controls, but was absent among AS patients. B*1402 has a widespread distribution, including among Caucasians, and has never been found associated with AS. Sequence analysis of B*1402-bound peptides (18) revealed a strong preference of this allotype for peptides with R2. Because the B pockets of B*1402 and B*1403 are identical, it was expected that this allotype would also bind peptides with this motif.

The availability of two closely related HLA-B14 molecules differentially associated with AS, allowed us for the first time to look for peptide differences that may correlate with disease susceptibility in a non-B27 system. It was also possible to look for similarities in peptide presentation between HLA-B27 and another relatively distant but AS-associated class I molecule, because B*1403 differs from B*2705 in 15 amino acid residues in the extracellular region of the molecule, all of which are located in the α1 and α2 domains. Thus, in this study we comparatively analyzed the peptide binding specificity of B*1402 and B*1403, to determine the overlap between their constitutive peptide repertoires and the
structural features of shared and differentially bound ligands. We also determined that peptide sharing between B*1403 and B*2705 is very low, although both allotypes bind some common ligands, and defined the molecular basis of their differential peptide specificity. Finally, allo-reactive CTLs, which recognize a wide spectrum of the peptides constitutively bound to the allo-MHC molecule (19–21), were used to analyze the relationship between epitope and peptide sharing among B*1402, B*1403, and B*2705.

EXPERIMENTAL PROCEDURES

Cell Lines and DNA-mediated Gene Transfer—HMy2.C1R (C1R) is a human lymphoid cell line with low expression of its endogenous HLA class I molecules (22, 23). B*2705-C1R transfectants with high expression of HLA-B27 were described elsewhere (24). C1R transfectant cells expressing B*1402 and B*1403 were obtained as follows. RNA was purified from the lymphoblastoid cell line (LCL) R56 (HLA-A2, A11; B*1402, B*2702). Full-length cDNA of B*1402 was obtained by PCR, using two HL A-B locus-specific primers hybridizing in the 5′-flanking and 3′-untranslated regions, respectively. The DNA fragment was cloned into the pCRII vector (Invitrogen), sequenced to confirm that it corresponded to the correct B*1402 sequence, subsequently cloned into pCDNAIII (Invitrogen), sequenced again, and transfected into C1R cells by electroporation at 250 V and 960 microfarads, as previously described (24). One single nucleotide change, at codon 156, differentiates B*1403 from B*1402 (CGG to CGG). The gene encoding B*1403 was obtained by PCR-mediated site-directed mutagenesis of the B*1402 cDNA obtained from R56 cells. Two independent PCR reactions were performed. In the first one, an oligonucleotide hybridizing in the 5′-end region and an “upstream” primer containing the desired mutation were used. The second PCR reaction used an oligonucleotide hybridizing in the 3′-untranslated region and a “downstream” primer with the mutation. A third PCR reaction was then performed using both DNA fragments and cDNA from R56. The full-length cDNA fragment obtained was cloned into pCRII and fully sequenced to confirm that it corresponded to the B*1403 gene. Finally, the gene was cloned into pCDNAIII and sequenced again before transfecting it into C1R cells as above. These cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal bovine serum (both from Invitrogen).

Monoclonal Antibodies—The mAbs used in this study were W6/32 (IgG2a, specific for a monomorphic HLA class I determinant) (25) and BBM1 (IgG2b, specific for human B*2705) (26).

Flow Cytometry—About 6 × 10⁶ untransfected or B*1402-, B*1403-, or B*2705-C1R-transfectant cells were washed twice in 200 μl of phosphate-buffered saline and resuspended in 50 μl of undiluted mAb supernatant. After incubating for 30 min, cells were washed twice in 200 μl of phosphate-buffered saline, resuspended in 50 μl of fluorescein isothiocyanate-conjugated anti-mouse IgG rabbit antiseraum (Calbiochem-Norabiochem GmbH, Schwalbach, Germany), incubated for 30 min, and washed twice in 200 μl of phosphate-buffered saline. All operations were done at 4 °C. Flow cytometry was done in an Epics Profile XL instrument (Coulter Electronics Inc., Hialeah, FL), using the System II software.

Isolation of B*1402-, B*1403-, and B*2705-bound Peptides—B27- and B14-bound peptides were isolated from about 10⁶ of the corresponding C1R transfectant cells as previously described (27). Briefly, cells were lysed in 1% Nonidet P-40 in the presence of a mixture of protease inhibitors. After centrifugation, cell lysates were subjected to affinity chromatography using the W6/32 mAb. HLA-B27- and HLA-B14-bound peptide pools were eluted with 0.1% aqueous trifluoroacetic acid at room temperature, filtered through Centricron 3 (Amicon, Beverly, MA), concentrated, and subjected to high performance liquid chromatography (HPLC) fractionation. This was conducted in a Waters Alliance system (Waters Milford, MA) using a Vydac 218TP52 column (Vydac, Hesperia, CA) at a flow rate of 100 μl/min, as previously described (28). Fractions of 50 μl were collected.

Mass Spectrometry Analysis and Sequencing—The peptide composition of HPLC fractions was analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS using a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker- Franzen Analytic GmbH, Bremen, Germany) equipped with the SCOUTTM source in positive ion reflector mode. Dried fractions were resuspended in 0.5 μl of 33% aqueous acetonitrile and 0.1% trifluoroacetic acid. This was deposited onto the stainless steel MALDI probe and allowed to dry at room temperature. Then 0.5 μl of matrix solution (α-cyano-4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid) at 1 mg/ml was added, and the mixture was allowed to dry again at room temperature.

Peptide sequencing was carried out by quadrupole ion trap nanoelectrospray MS/MS in an LCQ instrument (Finnigan TermoQuest, San Jose, CA), exactly as detailed elsewhere (29, 30). Some sequences were obtained using an Esquire 3000Plus ion trap mass spectrometer (Bruker) after online chromatographic separation of samples as follows. Samples were dissolved in Buffer A (0.5% acetic acid in water) and loaded onto a column (100 mm × 100 μm internal diameter) packed with 5 μm Kromasil C18 beads (EKA Chemicals, Bohus, Sweden) and fractionated in a Famos-Switchos-Ultimate chromatographic system (LCPacking, the Netherlands) with a 45-min linear gradient of 5–30% Buffer B (90% acetonitrile, 0.5% acetic acid in water) at 500 nl/min. MS/MS spectra were acquired by automatic switching between MS and MS/MS mode using dynamic exclusion. Some samples were analyzed using the Multiple Reaction Monitoring mode, which allows specific peptide masses to be selected. The interpretation of MS/MS spectra was done manually, but assisted by various software packages, including Mascot (Matrix Science Ltd., London) and MSProduct, a facility of the Protein Prospector package (31). In addition, the FASTA software (www.ncbi.nlm.nih.gov/ fasta33/index.html) was used to look for unambiguous matching of candidate peptide sequences with the human genome and proteome databases.

CTL—Alloreactive CTL clones directed against B*1402 were obtained from various unrelated HLA-B14-negative donors upon stimulation with the LCL LWAGS (HLA-A*3301, B*1402) as follows. About 10⁶ peripheral blood mononuclear cells (PBMCs) were stimulated in vitro with a mixture of 10² stimulator LCL and 10⁶ autologous PBMC feeder cells irradiated at 80 and 50 Gy, respectively. After a week cells were directly cloned by seeding serial dilutions of stimulated T cells in U-bottomed 96-well plates containing 2,000 irradiated stimulator LCL and 20,000 irradiated PBMCs per well in the presence of 30 units/ml recombinant interleukin-2 (a kind gift of Hoffmann-La Roche, Nutley, NJ). Cells in wells growing below the statistical limit of clonality were tested for HLA-B14 reactivity, using a standard ¹²⁵I release cytotoxicity assay against B*1402-C1R targets, and untransfected C1R cells as a negative control. Mixed lymphocyte cultures were grown in Iscove’s modified Dulbecco’s medium with Glutamax I (Invitrogen), supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin sulfate, and 0.05 mg/ml gentamicin (all from Sigma-Aldrich) and 14% fetal bovine serum (Invitrogen). T-cell clones were grown in the same medium and restimulated weekly in the presence of interleukin-2, as described above.

Alloreactive CTL clones directed against B*2705 were obtained by the same procedure, with the following modifications. PBMCs from
various unrelated HLA-B27-negative donors were stimulated in vitro with either the R15 (HLA-A3, B*2705, B*35) or LG15 (HLA-A32, B*2705) LCL for a week, re-stimulated with the alternative LCL in secondary mixed lymphocyte culture for another week, and cloned with the initial LCL by limiting dilution. Other previously described B*2705-specific CTL clones were also used (32).

Statistical Analysis—This was carried out by the χ² with Yates correction or the Fisher’s exact test, depending on the size of the samples being compared. p values of <0.05 were considered as statistically significant.

RESULTS

Large Disparity between the Constitutive Peptide Repertoires of B*1402 and B*1403—The peptide pools from both subtypes were isolated, after immunoaffinity purification with W6/32 and acid extraction, from the corresponding C1R transfectant cells, whose surface expression of B*1402 and B*1403 was very similar (Fig. 1). Both peptide pools were fractionated by HPLC in consecutive runs under identical conditions, and each of the peptide-containing fractions was analyzed by MALDI-TOF MS. The MS spectrum of each HPLC fraction from one subtype was compared with the correlative, previous, and following fractions from the other subtype, to take into account small shifts in the retention time of peptides that may occur even in consecutive chromatographic runs. Ion peaks with the same (±0.8) mass-to-charge (m/z) ratio in this comparison, were considered to reflect identical ligands of both subtypes. Ion peaks found in only one peptide pool were considered to reflect subtype-specific ligands. This strategy has been previously used to determine the overlap between HLA-B27 subtype-bound peptide repertoires in several studies from our laboratory (33, 34). Two independent comparisons were carried out, and the overlap between the B*1402 and B*1403 peptide repertoires was established as the mean of the two experiments (TABLE ONE). On the basis of this analysis B*1402 and B*1403 shared only ~32–35% of their peptide repertoires. The peptide size distribution in both pools was very similar and showed a Gaussian distribution typical of MHC class I-bound peptides (Fig. 2A), with an average molecular mass of 1113 and 1109 Da for B*1402 and B*1403, respectively. Moreover, both the average molecular mass (TABLE ONE) and size distribution (Fig. 2B) of subtype-specific ligands were very similar between both sets. These results indicate that, despite their close structural similarity, B*1402 and B*1403 bind largely distinct peptide repertoires, although this has little effect on peptide size.

Limited Peptide Sharing between HLA-B14 and HLA-B27—The B*1402- and B*1403-bound peptide repertoires were separately compared with that of B*2705, using the same strategy as for the two HLA-B14 subtypes. B*1402 and B*1403 differed from B*2705 in 88 and 85% of their peptide repertoires, respectively (TABLE TWO). This is a minimum estimation, because a comparison based on retention times and molecular mass becomes more inaccurate in reflecting peptide identity, the more different are the peptide pools compared. Indeed, of six peptide pairs showing the same retention time and molecular mass that were sequenced from both B*1403 and B*2705 (see below), 2 (33.3%) and 4 (66.7%) corresponded to identical and different peptides, respectively. If these results are extrapolated to the total peptide pool, the actual overlap of the B*2705-bound peptide repertoire with that of B*1403 would be (8 × 0.333)% = 2.7%, and the overlap of the B*1403-bound repertoire with that of B*2705 would be (15 × 0.333)% = 5%.

The average molecular mass of the total B*2705-bound peptides was significantly higher (1222 Da) than those of B*1402 or B*1403 ligands (1113 and 1110 Da, respectively), suggesting notorious differences in side-chain composition, peptide length or both between the B14 and B27-bound peptide repertoires (Fig. 2A). This was clear from the size distribution of the differential peptides between B*2705 and B*1402 or B*1403 (Fig. 2C): B14-specific peptides predominated in the lower mass range, and the opposite was true for B*2705-specific ligands. Peptides shared between B*2705 and B*1402 or B*1403 had an average molecular

### TABLE ONE

| Comparison of natural peptide ligands from B*1402 and B*1403 | Exp. 1 | Exp. 2 | Mean |
|-------------------------------------------------------------|-------|-------|------|
| B*1402 | B*1403 | B*1402 | B*1403 | B*1402 | B*1403 |
| Total peptides compared | 782 | 742 | 909 | 800 | 846 | 771 |
| Shared peptides | 319 (41%) | 319 (43%) | 225 (25%) | 225 (28%) | 272 (32%) | 272 (35%) |
| Differential peptides | 463 (59%) | 423 (57%) | 684 (75%) | 575 (72%) | 574 (68%) | 499 (65%) |
| Average mass of total peptides | 1113 Da | 1104 Da | 1112 Da | 1114 Da | 1113 Da | 1109 Da |
| Average mass of shared peptides | 1100 Da | 1100 Da | 1092 Da | 1092 Da | 1096 Da | 1096 Da |
| Average mass of differential peptides | 1122 Da | 1108 Da | 1119 Da | 1123 Da | 1121 Da | 1116 Da |
mass (1127 and 1145 Da, respectively) somewhat higher than the total B*1402 or B*1403 ligands and clearly below the total B*2705 ligands (TABLE TWO).

The number of ion peaks counted from B*1402 and B*1403 were consistently lower than those from B*2705 in all experiments (TABLE TWO). There are several possible explanations for this result. First, it might be a consequence of the higher surface expression of HLA-B27, relative to HLA-B14 subtypes (Fig. 1). However, this possibility was not supported by the fact that the global peptide yields of the B27- and B14-bound pools were similar (data not shown). Second, a significant fraction of the B14-bound peptides might not dissolve in the aqueous trifluoroacetic acid used for peptide extraction. This is also very unlikely, because trifluoroacetic acid is a very efficient solvent for short peptides, in the range of the MHC class I ligands, whose neat charge is always positive, because the acidic groups are uncharged at acidic pH. Third, a significantly higher number of B14 than B*2705 ligands might escape detection by MALDI-TOF MS due to lower positive charge. Although there is no obvious reason for this, we have not experimentally addressed this possibility and cannot formally rule it out. Finally, it is possible that the B14-bound peptide repertoire is actually smaller than that of B*2705. This might result from restricted residue usage at a large number of peptide positions among B*1402 and B*1403 ligands (see below).

Taken together, these results indicate that B*2705 shares with B*1402 and B*1403 only a small fraction of its constitutive peptide repertoire, and that it binds peptides with higher molecular mass. Our results are also consistent with the possibility that the B*2705-bound peptide repertoire is larger than those of B*1402 and B*1403.

**Peptide Specificity of HLA-B*1402, B*1403, and B*2705**

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**FIGURE 2.** Size distribution of B*1402-, B*1403-, and B*2705-bound peptides. A, size range of the peptide pools from the three allotypes. B, size distribution of the peptides differentially bound to B*1402 or B*1403. C, size distribution of the peptides differentially bound to B*1403 or B*2705. Data are means of two independent experiments.

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**TABLE TWO**

|                  | Exp. 1 | Exp. 2 | Mean  |
|------------------|--------|--------|-------|
| **B*1402/B*2705**|        |        |       |
| Total peptides compared | B*1402 | B*2705 | B*1402 | B*2705 | B*1402 | B*2705 |
| Shared peptides   | 90 (12%) | 90 (8%) | 114 (13%) | 114 (7%) | 102 (12%) | 102 (8%) |
| Differential peptides | 692 (88%) | 1042 (92%) | 795 (87%) | 1439 (93%) | 744 (88%) | 1241 (92%) |
| Average mass of total peptides | 1113 Da | 1216 Da | 1112 Da | 1227 Da | 1113 Da | 1222 Da |
| Average mass of shared peptides | 1136 Da | 1136 Da | 1118 Da | 1118 Da | 1127 Da | 1127 Da |
| Average mass of differential peptides | 1110 Da | 1223 Da | 1112 Da | 1236 Da | 1111 Da | 1230 Da |
| **B*1403/B*2705**|        |        |       |
| Total peptides compared | B*1403 | B*2705 | B*1403 | B*2705 | B*1403 | B*2705 |
| Shared peptides   | 125 (17%) | 125 (11%) | 95 (12%) | 95 (6%) | 110 (15%) | 110 (8%) |
| Differential peptides | 614 (83%) | 1007 (89%) | 681 (88%) | 1458 (94%) | 648 (85%) | 1233 (92%) |
| Average mass of total peptides | 1105 Da | 1216 Da | 1115 Da | 1227 Da | 1110 Da | 1222 Da |
| Average mass of shared peptides | 1132 Da | 1132 Da | 1157 Da | 1157 Da | 1145 Da | 1145 Da |
| Average mass of differential peptides | 1100 Da | 1227 Da | 1109 Da | 1232 Da | 1105 Da | 1230 Da |
analogous residues (i.e. acidic, basic, polar, aliphatic, or aromatic) appeared at that position in at least 40% of the nonamers from a given subtype (Fig. 4). On this basis B*1402 and B*1403 showed skewed residue usage at 6 and 8 of the 9 peptide positions, respectively. Both subtypes had 4 positions with the same predominant motifs: P2 (Arg), P5 (Arg), P8 (polar residues), and P9 (aliphatic residues, mainly Leu). Except P9, which was fully restricted to aliphatic/aromatic residues, the other positions tolerated multiple residue types. Both subtypes had also in common a lack of significant restrictions in residue usage at P6. Due to the contribution of subtype-specific peptides, whose differential features are analyzed below, both subtypes showed differences in residue usage at 4 positions: P1, P3, P4, and P7. B*1402 showed no predominant (≥40%) motif at P1 and P4, and aliphatic motifs at P3 and P7. In contrast, B*1403 showed an acidic motif at P1, a statistically increased frequency of Asp at P3, although this was slightly lower than 40% and aliphatic residues were also allowed at this position, an aliphatic motif at P4, and a polar one at P7. However, due to the large contribution of shared ligands in the 2 series compared, the differences in residue frequencies were not statistically significant, except at P3 and P7. The peptide motifs of B*1402 were also analyzed in a previous report (18). In that study, an enrichment of particular residues at P1 (acidic), P2 (Arg), P3 (aliphatic/aromatic), P5 (Arg), P6 (Leu/Ile), and P7 (Leu) was found.

**FIGURE 3.** Amino acid sequence of B*1402 and B*1403 ligands. These are classified as shared, B*1402-specific, and B*1403-specific. Within each category they are listed by size and alphabetical order. The human proteins with which total match was obtained and their accession numbers in the Fasta databases (www.ebi.ac.uk/fasta33/index.html) are given. The one or more subtypes from which the peptides were sequenced are indicated. Those peptides sequenced from only one subtype but showing an ion peak with the same retention time and m/z in the corresponding MALDI-TOF MS spectrum from the other subtype are listed as shared ligands. A shared octamer previously reported as a B*1402 ligand (18) is indicated with an asterisk.
FIGURE 4. Peptide motifs of B*1402 (white bars) and B*1403 (black bars) ligands. Residue frequencies at each position among the sequenced nonamers from each subtype (Fig. 3) are indicated both for individual residues and for residue types (acidic: Asp and Glu; basic: His, Lys, and Arg; polar: Asn, Gln, Ser, and Thr; aliphatic: Ala, Cys, Ile, Leu, Met, and Val; aromatic: Phe, Trp, and Tyr; other: Gly and Pro). Residues or residue types showing a frequency ≥40% are indicated and assigned as peptide motifs. Residues showing lower frequency but a statistically significant difference between subtypes (Asp-3 and Thr-7 in B*1402) are also indicated. Statistically significant differences in residue frequencies among the nonamers from both subtypes (p < 0.05) were assessed with the Fisher's exact test. NS, not significant.
Our results are in agreement with these assignments, except for the restrictions at P6, which were not confirmed by our analysis.

**Structural Features of Shared B*1402/B*1403 Ligands**—The same analysis of residue frequencies was carried out for only the subset of 11 shared nonamers (Fig. 5). As expected, those positions that had a similar type of restriction (P2, P5, P8, and P9) or were unrestricted (P6) in both subtypes showed a very similar pattern among shared nonamers. In those positions that showed different residue usage among subtypes, a mixed pattern was observed in the subset of shared ligands. As in B*1402, P1 was unrestricted and P3 showed an aliphatic motif. As in B*1403, an aliphatic motif emerged at P4. At P7, where both subtypes showed distinct (aliphatic/polar) restrictions, the subset of shared ligands showed no predominant motif. Overall, shared ligands showed restricted heterogeneity at 6 of 9 peptide positions, the most conspicuous motif being at P9.

**Subtype-specific HLA-B14 Ligands Have Distinct Motifs at Multiple Positions**—The basis for the disparity between the B*1402- and B*1403-bound peptide repertoires was analyzed by comparing the structural features of subtype-specific ligands. A comparison of residue frequencies between the corresponding nonamers revealed a number of significant differences (Fig. 6). P1 was mainly restricted to acidic residues among B*1403-specific nonamers but not among the B*1402-specific set. P2 was restricted to Arg and Gln among B1402-specific nonamers and was more degenerate in the B*1403-specific set. However, the differences in these two positions did not reach statistical significance. A very dramatic difference was observed at P3, were Phe or aliphatic residues and Asp were the predominant motifs among B*1402- and B*1403-specific nonamers, respectively. Both differences were statistically significant. At P4 aliphatic residues were statistically increased among B*1403-specific nonamers. Another drastic difference was observed at P7: aliphatic residues, mainly Leu, were predominant in the B*1402-specific set, whereas a polar motif, mainly Thr, was observed in B*1403, both showing statistical significance. The increased abundance of polar residues at P8 in B*1402, relative to B*1403, was not statistically significant. At P9 a full restriction to nonpolar residues was observed in both sets. Among B*1402-specific nonamers, only aliphatic residues, mainly Leu, were found, whereas in the B*1403-specific set Phe was also frequent (50%). These differences were not statistically significant.

Thus, the L156R change between B*1402 and B*1403 modulates peptide specificity by altering residue preferences in at least three peptide positions: P3, P4, and P7. The differences in residue frequencies observed at other positions (P1, P2, P5, P8, and P9) might reflect altered residue preferences or result just from the small number of peptide sequences available for comparison.

**Differential Features between HLA-B*1402, B*1403, and B*2705 Ligands**—A total of 13 peptides (2 octamers, 10 nonamers, and 1 decamer) found in B*1403 but not in B*2705 were sequenced in this comparison (Fig. 7). All these peptides were sequenced either as shared or as B*1403-specific ligands in the B*1402/B*1403 comparison (Fig. 3). In addition, 8 B*2705 ligands (4 nonamers and 4 decamers) not found in B*1403 were also sequenced. Of these, 5 were previously identified HLA-B27 ligands (7) and 3 are reported here for the first time (Fig. 7). The basis for the large disparity between the B*1403- and B*2705-bound peptide repertoires was analyzed by means of a statistical com-
FIGURE 6. Differential motifs of B*1402-specific (white bars) and B*1403-specific (black bars) nonamers. Residue frequencies at each position among the sequenced nonamers from each subtype (Fig. 3) are indicated both for individual residues and for residue types (see legend to Fig. 4). Residues or residue types with a frequency ≥50%, and those positions showing statistically significant differences between both sets are indicated. Statistical significance (p < 0.05) was assessed with the Fisher’s exact test. NS, not significant.
Comparison of residue usage between the sequenced B*1403-bound and a previously published registry (7) of 108 B*2705-bound nonamers (TABLE THREE). The whole set of 19 sequenced B*1403 nonamers, including both shared ligands with B*1402- and B*1403-specific peptides (Fig. 3), were analyzed. Statistically significant differences in residue usage between B*1403 and B*2705 were found in most positions, except P4 and P6. Thus, at P1 acidic residues and R/H predominated in B*1403 and B*2705, respectively. At P2 B*2705 was clearly more selected.

### TABLE THREE

**Statistical differences in residue usage between B*1402, B*1403, and B*2705-bound nonamers**

The 16 and 19 sequenced nonamers from B*1402 and B*1403, respectively (Fig. 3) were compared with a published registry of 108 natural B*2705-bound nonamers (7). Statistical differences (p < 0.05) were determined by the χ² test with Yates correction.

| Position | statistically different residues, B*1402 | statistically different residues, B*1403 | statistically different residues, B*2705 | p       |
|----------|------------------------------------------|----------------------------------------|------------------------------------------|---------|
| P1       | Glu: 6                                   | Glu: 11                                | Arg + Gly: 0                             | 6.07 × 10⁻⁶ |
| P2       | Gln: 19                                  | Gln: 11                                | Gln: 1.9                                 | 0.012   |
| P3       | Gln: 19                                  | Gln: 11                                | Gln: 1.9                                 | 3.54 × 10⁻¹¹ |
| P4       | Gln: 19                                  | Gln: 11                                | Gln: 1.9                                 | 5.64 × 10⁻¹⁸ |
| P5       | Gln: 19                                  | Gln: 11                                | Gln: 1.9                                 | 3.54 × 10⁻⁸ |
| P6       | Gln: 19                                  | Gln: 11                                | Gln: 1.9                                 | 5.62 × 10⁻¹⁸ |
| P7       | Gln: 19                                  | Gln: 11                                | Gln: 1.9                                 | NS      |
| P8       | Gln: 19                                  | Gln: 11                                | Gln: 1.9                                 | 0.014   |
| P9       | Gln: 19                                  | Gln: 11                                | Gln: 1.9                                 | 0.003   |
| Basic: 0 | Basic: 0                                 | Basic: 0                               | Basic: 0                                 | 0.046   |

a Percent frequency of residues with statistically different usage between B*1402- or B*1403- and B*2705-bound nonamers. Statistically increased frequencies and the corresponding residues are in boldface.

b p values corresponding to the B*1402/B*2705 and B*1403/B*2705 comparisons, respectively. For the B*1402/B*1403 comparison see Fig. 4.

c NS, not significant.
The HLA class I types of the donors are as follows: Donor 1 (HLA-A1, A2; B7, B18; Cw5, Cw7); Donor 2 (HLA-A2, A23; B44, B62; Cw2, Cw4); Donor 3 (HLA-A23, 24; B51, B*1503; Cw4, Cw5); and Donor 4 (HLA-A2, A29; B44, B57, Cw7).

**Table FOUR**

| Natural ligands shared between HLA-B27 and HLA-B14 | B*2705 | B*1402 | B*1403 | Other B27 subtypes |
|--------------------------------------------------|--------|--------|--------|--------------------|
| Peptide                                          |        |        |        |                    |
| GRVGEFFPRSY                                      | This study and Ref. 7 | Yes    | Yes    |                    |
| IRAAPPPLF                                        | This study and Ref. 7 | Yes    | Yes    |                    |
| LRFPGQLNA                                        | This study | Yes    | Not found |                    |
| SRFPEALRL                                        | (7)     | Yes    | Not found | B*2706 (7)        |

*a* See Fig. 7.  
*b* See Fig. 3.

**Table FIVE**

| Cross-reaction among B*1402, B*1403 and B*2705 with alloreactive CTL |
|---------------------------------------------------------------------|
| **α-B*1402 CTL**                                                   | **Relative lysis with B*1403** | **CTLs cross-reactive with B*1403** |
| **Donor** | **Number of CTLs** | <30% | 30–70% | >70% | |
| 1          | 42            | 30 (71.4%) | 9 (21.4%) | 3 (7.1%) | 12 (28.6%) |
| 2          | 23            | 16 (69.6%) | 4 (17.4%) | 3 (13%) | 7 (30.4%) |
| 3          | 3             | 2 (66.7%)  | 0       | 1 (33.3%) | 1 (33.3%) |
| 4          | 6             | 5 (83.3%)  | 1 (16.7%) | 0       | 1 (16.7%) |
| **Total**  | 74            | 53 (71.6%) | 14 (18.9%) | 7 (9.5%) | 21 (28.4%) |

| **α-B*1402 CTL**                                                   | **Relative lysis with B*2705** | **CTLs cross-reactive with B*2705** |
| **Donor** | **Number of CTLs** | <30% | 30–70% | >70% | |
| 1          | 42            | 42 (100%)  | 0       | 0       | 0        |
| 2          | 22            | 20 (90.9%) | 1 (4.5%) | 1 (4.5%) | 2        |
| 3          | 3             | 3 (100%)   | 0       | 0       | 0        |
| 4          | 3             | 3 (100%)   | 0       | 0       | 0        |
| **Total**  | 70            | 68 (97.1%) | 1 (1.4%) | 1 (1.4%) | 2 (2.9%) |

*a* The HLA class I types of the donors are as follows: Donor 1 (HLA-A1, A2; B7, B18; Cw5, Cw7); Donor 2 (HLA-A2, A23; B44, B62; Cw2, Cw4); Donor 3 (HLA-A23, 24; B51, B*1503; Cw4, Cw5); and Donor 4 (HLA-A2, A29; B44, B57, Cw7).

Alloreactive CTL Cross-reaction among B*1402, B*1403, and B*2705

**Parallels Peptide Sharing**—Alloreactive CTL responses are characterized by large clonal diversity (35). This is mainly due to the fact that the CTL clones recognize a vast array of the peptides constitutively bound to the allo-MHC molecule (19–21). Thus, determining the cross-reactions of allospecific CTL allowed us to analyze the shared antigenic features of related allo-MHC molecules as a function of their constitutive peptide repertoires.

A total of 74 alloreactive CTL clones elicited against B*1402 from various donors were tested for their capacity to lyse B*1403-CEG-transfected cells (TABLE FIVE). The specificity of the CTL clones was established from their lack of killing of untransfected C1R cells and their lysis of B*1402-CEG transfected. Cross-reactivity was assessed as the lysis of B*1403 target cells, relative to the lysis of B*1402-CEG-transfected cells. Relative lysis values lower than 30% were considered as partial cross-reaction, indicating full conservation of the allospecific epitope. Relative lysis values >70% were considered as full cross-reaction, indicating full conservation of the allospecific epitope. Relative lysis values of 30–70% were considered as partial cross-reaction, indicating a partially altered epitope as a consequence of either diminished presentation or altered conformation of the corresponding allospecific peptide. Only 21 of the 74 anti-B*1402 CTL clones tested (28.4%) showed either partial or full cross-reaction with B*1403, with little difference among donors. This percentage was only slightly lower than the percentage of peptide overlap between B*1402 and B*1403 (32–35%). Because these CTLs recognize constitutive B*1402-bound peptides, these results suggest that, globally, many of the shared ligands between B*1402 and B*1403 maintain their antigenic features, presumably by adopting a similar conformation, when bound to both subtypes.

A similar analysis was performed to analyze allospecific epitope sharing between HLA-B14 and B*2705. For this purpose we tested alloreactive...
Peptide Specificity of HLA-B*1402, B*1403, and B*2705

tive CTL clones raised against B*1402 for recognition of B*2705-C1R targets. Anti-B*1403 CTL could not be obtained, because B*1403-positive LCL, to be used as stimulators, were not available, and attempts to raise alloreactive CTL using B*1403-C1R as stimulator cells were unsuccessful. Of a total of 70 anti-B*1402 CTL from four donors, only 2 (2.9%), both from the same donor, showed either partial or full cross-reaction with B*2705. This percentage was very close to the estimated peptide sharing between B*1403 and B*2705, when the percent overlap between both allotypes obtained by MALDI-TOF (8–15%) was corrected for the percentage of shared ion pairs confirmed as identical peptides by sequencing (2 of 6: 33.3%), resulting in an estimated peptide overlap between B*1403 and B*2705 of 2.7 to 5% (see above). Although a similar analysis was not performed with B*1402, it is reasonable to infer that the B*1402/B*2705 overlap might be similar, because their peptide sharing by MALDI-TOF MS (8–12%) was very similar to the B*1403/B*2705 overlap.

The two anti-B*1402 CTLs that cross-reacted with B*2705 showed marginal cross-reaction (27 and 30% relative lysis, respectively) with B*1403, suggesting that they recognized peptides shared by B*1402 and B*2705, but absent or antigenically altered in B*1403. Two peptides with such features were identified in this study (TABLE FOUR).

In addition, 28 and 20 alloreactive CTL clones obtained from various donors against B*2705 were tested for recognition of B*1402 and B*1403, respectively. None of these CTL lysed the B14 targets (data not shown). Given the low peptide sharing, it is possible that the number of anti-B*2705 CTL tested was not sufficient to detect cross-reaction with HLA-B14.

DISCUSSION

The B*1402 and B*1403 subtypes showed a disparity in their constitutive peptide repertoires that was strikingly large in view of their close structural similarity, with only an L156R change. For instance, B*4402 and B*4403, which differ from each other only by the D156L change, share >95% of their peptide repertoires (36). In principle, polymorphism at residue 156 may influence peptide specificity through at least two mechanisms. First, by directly modulating peptide binding, because this residue takes part in the D and E pockets of the peptide-binding site, which accommodate the peptideic P3 and P7 residues, respectively (9, 37). Second, by modulating peptide loading through interactions with the peptide-loading complex. Residue 156 was suggested to influence the interaction with the transporter associated with antigen processing (TAP), which is mediated by the MHC-dedicated chaperone tapasin (38), on the basis of the different TAP binding efficiency of B*4402 and B*4403 (39, 40). This mechanism is not supported by the high peptide sharing between the two B44 subtypes (36), but it does not exclude a different effect of the B*1402/B*1403 dimorphism on TAP-mediated peptide loading, because the amino acid change at position 156 in these subtypes is different. Although interactions with the peptide-loading complex were not addressed in this study, the peptide differences between B*1402 and B*1403 can be explained to a large extent by an effect of residue 156 on peptide-binding specificity. Statistically significant differences in residue usage among differentially bound peptides were found at three positions two of which, P3 and P7, bind in the pockets of which residue 156 is an integral part. The preference for nonpolar residues at both positions in B*1402 and for acidic (Asp) and polar (mainly Thr) residues at P3 and P7, respectively, in B*1403 are fully consistent with the hydrophobic Leu-156 in B*1402 and the basic Arg-156 in B*1403. The basis for the increased preference of B*1403 for aliphatic P4 residues is not obvious in the absence of an x-ray diffraction model. It might result from readjustments in side-chain orientation and contacts in the vicinity of residue 156, indirectly influencing the binding mode and frequency of given P4 residues.

For B*1402, acidic P1 residues were suggested to be favored by the presence of Asn-63 (18). An increased preference of B*1403 for this type of residues was evident from the sequences of subtype-specific ligands, although it did not reach statistical significance. This increase is also not readily explained by a direct effect of Arg-156, because B*1402 and B*1403 have the same A pocket, including the Asn-63 residue. It is possible that indirect, long-range effects of residue 156 on residues close to the A pocket might modulate P1 residue specificity between B*1402 and B*1403, but this can only be properly addressed by crystallographic analysis. Alternatively, the preference of B*1403 for acidic P1 residues might reflect lower TAP dependence of this allotype, because these, along with acidic P3 residues, are among the most disfavored motifs for TAP binding (41–44). Of the eight B*1403-specific nonamers sequenced in this study, five had acidic residues at both P1 and P3, and all but one had these types of residues in at least one of these positions. These results might have two explanations. A first possibility is that B*1403 interacts poorly with proteins in the peptide-loading complex and is more permissive than B*1402 for peptides with disfavored TAP-binding motifs. A second possibility is that the increased preference for acidic P3 residues, resulting from a direct influence of Arg-156 on Asp pocket interactions, helps to bypass TAP binding requirements for peptide loading, indirectly favoring binding of peptides that also have acidic P1 residues, without involving suboptimal interactions of B*1403 with other components of the peptide-loading complex. This issue can be addressed by direct analysis of these interactions, which was not pursued in this study. In addition, because many MHC class I ligands enter the endoplasmic reticulum as N-terminally extended precursors and are then subjected to aminopeptidase-mediated trimming (45, 46), the relationship between N-terminal TAP-binding motifs of HLA class I ligands and TAP dependence of the corresponding allotype is not straightforward. However, it is interesting that one of the shared ligands among B*1402, B*1403, and B*2705, IRAAPPFLF, which derives from the signal peptide of cathepsin A, is a TAP-independent ligand, as confirmed by its isolation from the B*2705-bound peptide pool from the TAP-deficient T2 cells.3 Thus, the significant quantitative and qualitative differences between the B*1402- and B*1403-bound peptide repertoires are largely explained by the concomitant influence of residue 156 on at least two anchor positions of the bound peptides, although additional effects on TAP dependence cannot be formally excluded.

Aside of other putative differences between these two subtypes, which remain to be investigated, such as interaction with the peptide-loading complex, folding kinetics, tendency to misfold, or the capacity to form heavy chain homodimers, it seems clear that the large differences between their peptide repertoires, allow much room for the possibility that the differential association of B*1402 and B*1403 to AS may be related to their differential peptide specificity, as proposed by the arthritogenic peptide hypothesis (47). In principle, an additional level of disparity might arise from distinct presentation of shared ligands by these two subtypes, as observed in HLA-B44 (36). To test this issue we used alloreactive CTL clones as probes to analyze peptide-dependent epitope sharing between B*1402 and B*1403. This approach was based on three experimentally well supported assumptions. First, that alloreactive CTLs recognize constitutive alloantigen-bound peptides (19–21). Second, that the large clonal diversity of alloreactive CTL responses is due to recognition of a vast array of peptides constitutively bound to the alloantigen molecule, so that virtually every CTL clone recognizes a

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3 M. Ramos and J. A. López de Castro, unpublished observations.
different peptide (19, 21). This is unlike self-restricted CTL responses, where the majority of the CTL clones are directed against one or few immunodominant foreign peptides. Third, although CTLs can cross-react with very distinct peptides showing minimal sequence homology among each other (48), it is reasonable to assume that alloreactive CTL cross-reaction between closely related allotypes with a substantial degree of peptide sharing will be generally based on recognition of shared ligands. This assumption is very difficult to substantiate experimentally because, in general, the allospecific peptide recognized by any given CTL clone is unknown. However, it was formally demonstrated for HLA-B*27 subtypes in at least one instance (27). Furthermore, at least some shared ligands of B*2705 and B*2709 show a highly similar crystal structure when bound to both allotypes (49, 50). Thus, the finding that the percentage of allospecific CTL clones cross-reacting between B*1402 and B*1403 closely reflected peptide sharing, strongly suggests that most of the shared ligands maintain their antigenic features, and presumably a similar conformation, when bound to either subtype. Our results with allore cognition of HLA-B14 subtypes are different from those reported for B*4402 and B*4403. In this subtype pair, the D156L change had a limited effect on peptide specificity, and a drastic one on allore cognition (36). A different mutation in the same position of HLA-B14 (L156R) has significant effects on peptide specificity, but seems to have little effect on allore cognition of shared ligands. Thus, the effects of subtype polymorphism in a given position cannot be generalized, because they may depend strongly on the nature of the mutation, the molecular context in which it occurs, or both. For instance, B*3508 and B*3501, which also differ by only the L156R change, bind a shared peptide epitope with a similar conformation, but immunodominance and T-cell receptor engagement of this epitope was subtype-dependent due to an effect of this polymorphism on the conformation of the peptide-binding site (51).

If susceptibility to AS were indeed dependent on T-cell recognition of specific peptides, as proposed by the arthritogenic peptide hypothesis (5), one would expect to find shared ligands and common peptide features between the disease-associated B*2705 and B*1403. The existence of shared ligands between these two allotypes was formally confirmed in this study. However, it is obvious that sharing common ligands with HLA-B27 does not, by itself, imply that the corresponding allotype will predispose to AS. This is best illustrated by the lack of association of B*2706 and B*2709 with this disease, despite their large peptide sharing with B*2705 and B*1402. Despite the low number of shared ligands, it was remarkable that some T-cell cross-reaction between B*1402 and B*2705 could be detected, and that the percentage of cross-reactive CTL clones was comparable with the low peptide sharing between B*1402 or B*1403 and B*2705. Thus, regardless of the many amino acid differences between HLA-B27 and HLA-B14, some of the shared ligands may maintain their alloantigenic features on both allotypes. Alternatively, these cross-reactions might reflect recognition of distinct peptides on each allotype, but this would imply that different ligands of HLA-B27 and HLA-B14 show antigen mimicry, therefore compensating their biochemical distinctness with their immunological similarity. Although anti-B*1403 CTLs were not analyzed, it seems reasonable to infer that a similar level of CTL cross-reaction may occur between B*1403 and B*2705. The joint finding of few shared ligands and cross-reactive CTL clones between HLA-B27 and HLA-B14 suggests that B*1403 and B*2705 present either some shared peptides with the same antigenic features or distinct peptides showing antigen mimicry. This is a basic requirement of the arthritogenic peptide hypothesis (5). However, our results are based on allor eactive CTLs, which are obviously unrelated to spondyloarthritis, and formal proof of pathogenetically relevant antigen mimicry between B*1403 and B*2705 ligands would require confirmation with self-restricted CTLs.

Two other relevant aspects should be considered. First, no peptides shared by B*1403 and B*2705, but absent in B*1402, were found. Although our results do no exclude the existence of such ligands, they must be rare. Second, the basis for the large differences between the B*1403- and B*2705-bound peptide repertoires are clearly explained by the very distinct requirements of the respective peptide ligands at multiple positions, and by the fact that many residues favored for one allotype are rarely or not found among ligands of the other. These findings do not exclude the existence of a common disease-related peptide presented by B*1403 and B*2705, but not by B*1402, but suggest that the likelihood of finding such a peptide may be low.

In conclusion, our study has defined biochemical and immunological relationships between two HLA class I allotypes associated with AS that may set limitations to some assumptions concerning the pathogenesis of this disease, such as involvement of specific peptides. However, molecular correlations among peptide repertoires and the use of alloreactive CTLs that, although useful as probes for the antigenic conservation of shared ligands bound to different MHC molecules are unrelated to AS, cannot provide a direct answer to the mechanism of spondyloarthritis. Ultimately, the pathogenic features of HLA-B27 and B*1403 need to be identified through further studies in patients.

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Peptide Specificity of HLA-B*1402, B*1403, and B*2705

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Two HLA-B14 Subtypes (B*1402 and B*1403) Differentially Associated with Ankylosing Spondylitis Differ Substantially in Peptide Specificity but Have Limited Peptide and T-cell Epitope Sharing with HLA-B27

Elena Merino, Verónica Montserrat, Alberto Paradela and José A. López de Castro

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