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The Down-Regulation of HLA-DM Gene Expression in Rheumatoid Arthritis Is Not Related to Their Promoter Polymorphism

Pascale Louis-Plence,* Sophie Kerlan-Candon,† Jacques Morel,‡ Bernard Combe, Jacques Clot,* Valérie Pinet,* and Jean-François Eliaou**

HLA-DM molecule, a class II-like heterodimer, is a critical factor of HLA class II-dependent Ag presentation. It acts as a molecular chaperone and also functions as a peptide editor favoring the presentation of high-stability peptides. Thus, it appears to skew the peptide repertoire presented to T cells. Variation in HLA-DM expression has considerable effect on Ag presentation and regulation of these genes is likely to be a prerequisite to prevent autoimmunity. In this study, rheumatoid arthritis (RA) was chosen as a model of human autoimmune disease since its genetic susceptibility is known to be associated with the HLA-DR and -DM components. We described a limited nucleotide polymorphism in the HLA-DM promoters with functional impact on basal transcriptional activity and IFN-γ induction as assessed in vitro. However, no difference of allele frequencies was found between controls and RA patients. Despite of this lack of association, expression of HLA-DM molecules was also investigated. Interestingly, an underexpression of HLA-DM transcripts and protein was shown in peripheral blood B cells from RA patients compared with controls or inflammatory arthritis patients. This underexpression does not affect HLA-DR genes and is responsible for a decrease of the DM:DR ratio in RA patients. This specific HLA-DM down-regulation is likely to have important consequences on Ag presentation and could participate in the autoimmune process in RA. The Journal of Immunology, 2000, 165: 4861–4869.

Major histocompatibility complex class II molecules are peptide receptors involved in the presentation of self and antigenic peptides present in the endocytic pathway to CD4+ T cells (1). MHC class II molecules typically assemble in the endoplasmic reticulum as a stoichiometric complex with a nonpolymorphic chain, the invariant chain (Ii)5 (2). The nonameric complex (αβIi), is transported to the trans-Golgi network and subsequently sorted to endosomal/lysosomal compartments (3, 4). In this acidic environment, Ii is proteolytically degraded and the ultimate product of degradation, class II-associated Ii peptide (CLIP), forms intermediate complexes with αβ dimers. Then CLIP is replaced by antigenic peptides before the transport of the MHC class II molecules to the cell surface can proceed (5). Although CLIP by itself can dissociate from several HLA class II alleles at endosomal pH (6–8), another class II-like heterodimer, HLA-DM, appears to be required for efficient CLIP release and acquisition of cognate peptides for all class II alleles (9, 10).

HLA-DM molecule catalyzes the exchange of CLIP for cognate peptide in an enzyme-like fashion but is not restricted to class II-CLIP complexes and exhibits a rather broad substrate specificity (11–14). Indeed, HLA-DM was found to release non-CLIP peptides that formed with αβ dimers a low-kinetic stability complex, owing to suboptimal anchor-pocket interactions or suboptimal length. After removal of CLIP or low-intrinsic kinetic stability peptides, HLA-DM acts as a molecular chaperone and keeps empty HLA class II-binding grooves accessible for cognate peptide at low lysosomal pH so that loading can be accomplished even when a low amount of peptide is available (15). Therefore, HLA-DM acts as a peptide editor (13) and appears to skew the peptide repertoire presented to T cells toward a population of high-kinetic stability MHC class II-peptide complexes. Thus, HLA-DM reduces the diversity of the set of antigenic peptides exposed at the cell surface and may contribute to the selection of immunodominant epitopes. In the absence of HLA-DM expression, the peptide repertoire bound to HLA class II molecules could be broader, leading to the presentation of new epitope determinants triggering the activation of autoreactive T cells. Thus, variation in HLA-DM expression has considerable effect on the peptide repertoire bound to HLA class II molecules and presented to CD4+ T cells (16–19), and regulation of HLA-DM expression appears to be a prerequisite to prevent autoimmunity.

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Abbreviations used in this paper: Ii, invariant chain; CLIP, class II-associated Ii peptide; RA, rheumatoid arthritis; AS, ankylosing spondylitis; wt, wild type.
consequences on their transcriptional activities and DNA-binding affinities (23–26). To explore nucleotide polymorphism, we sequenced the HLA-DM and DMB regulatory regions, and we investigated the functional consequences of the observed substitutions on transcriptional activity, IFN-γ induction, and affinity for the trans-acting NFκB factor.

Dysregulation of HLA class II molecule expression has been described in several autoimmune diseases and particularly in rheumatoid arthritis (RA) (27, 28). Genetic studies have demonstrated that some HLA-DR (29, 30) and HLA-DM (31) alleles are associated with the disease but the mechanism by which these alleles mediate susceptibility to RA is still unknown. According to the coordinate regulation of HLA class II genes and the genetic association between DM and RA, we compared the level of HLA-DM gene expression in physiology and RA patients. We quantified by semiquantitative RT-PCR the HLA-DM and -DMB transcripts and by Western blot analysis the HLA-DM protein level. We were able to show an underexpression of HLA-DM transcripts and protein in peripheral blood B cells from RA patients. This downregulation was restricted to the HLA-DM genes, suggesting a noncoordinate expression of DM and DR gene expression in RA. An arbitrary DM:DR ratio was calculated and was found to be three times decreased in RA patients compared with controls.

Materials and Methods

Direct sequencing of the HLA-DM proximal promoter region

One hundred six healthy volunteer bone marrow donors and 96 RA patients with classical disease according to the American College of Rheumatology criteria were studied. Genomic DNA was extracted from PBMC with the QIAamp Blood kit from Qiagen (Courtaboeuf, France). The nucleotide sequences of the HLA-DM and -DMB regulatory regions were determined following PCR amplification of genomic DNA. The nucleotide sequences of the oligonucleotides used were: sense, 5′-AGATGCGGTTCCCCATTG-3′; antisense, 5′-ATCTGCAAGAAAGAATATG-3′, antisense, 5′-TGCTCTGCTCTGTAAGATGC-3′ for HLA-DM, and sense, 5′-ATCTGCAAGAAAGAATATG-3′, antisense, 5′-TGCTCTGCTCTGTAAGATGC-3′ for HLA-DMB. The −21M13 primer sequence (TGTTAAACGACGGCAGCAG) was added 5′ to one of the two oligonucleotides in each set, and alternatively on the sense and antisense primers to perform the sequencing of the PCR amplified fragment, on both strands, using the ABI PRISM Dye Primer kit from Applied Biosystems (PE France SA, Courtaboeuf, France). The sequencing reactions were analyzed on an Applied Biosystems 373A DNA automatic sequencer. Each nucleotide substitution was confirmed by sequencing two independent PCR products as well as sequencing the complementary strand of DNA. The sequence Navigator software (PE France SA) was used to perform sequence alignments with the reference sequences found in the database (32).

Semi quantitative and competitive RT-PCR

B cells were purified from fresh peripheral blood with anti-CD19 mAb-coated magnetic beads from Dynal (Compiègne, France) and used directly for RNA extraction. Total RNA was extracted with an RNeasy kit from QIAGEN (Hilden, Germany). The nucleotide sequences of the primers used for the HLA-DM PCR were 5′-TGCTCTGCTCTGTAAGATGC-3′ for each RNA sample and the PCRs were conducted with increasing quantities of cDNA and were quantified using a luciferase assay system kit (Promega, Charbonnieres, France) in a TD20 luminometer counter (Promega) following the manufacturer’s procedure. The transcriptional activity of each construction was tested in five independent transfection experiments. Two plasmid preparations from two different clones for each sequence were used and the relative luciferase activities were compared.

Oligonucleotides for EMSA

The oligonucleotides used were as follows (κB motifs are in boldface, the mutated nucleotides used to disrupt the binding sites are underlined, and the polymorphic nucleotides in the κB site of the HLA-DM promoter region are in italics): κB IL-2κBα, CAACAGGGAAGGGAACTCTGCCTCCCTCTCT; κB IL-2κBα, CAACACTGAAGTCATCCTGCCTCTCTCTCCT; DMB-C, ATATAGGCGGTTGCTATCCCGCCCACTACTATT; GCGG-GT; DM-CT, ATATACGGGCTCTGATTACACCTACTACTCTATTTGGG; non-specific DNA competitor, TTGGAGACACCTTTGCCCTTGAAGATGCGTCTCTGTAAGATGC-3′ (antisense). The sequence of the primers used for GAPDH amplification were 5′-GGATTTGGTCGTATTGTGGCAAGAAGGTATGGGT-3′ (sense) and 5′-GGATTTGGTCGTATTGTGGCAAGAAGGTATGGGT-3′ (antisense). All of the PCRs were performed essentially as described previously (33). Amplified fluorescent PCR fragments were electrophoresed for 5 h on an Applied Biosystems 373A DNA and separated according to their size products (158 bp for DNA, 144 bp for DMB, and 166 bp for GAPDH).

Peaks of fluorescence were displayed using the Genescan software (Version 673; PE France SA) and intensities of fluorescence, corresponding to the calculated area of each peak, were determined. The signal for HLA-DM transcripts was then related to the signal obtained for GAPDH transcripts determined in the same way to normalize yields of RNA extraction and reverse transcription between samples.

For quantitative evaluation of the HLA-DRB mRNA expression, the sequences of the primers and the conditions for the competitive PCR have previously been described in detail (33).

Statistical analysis

Allelic frequencies were compared in Fisher’s exact test, values for HLA-DM:GAPDH and DM:DR ratios were compared according to the unpaired t test.

Cells and cell culture

Daudi is a MHC class II-positive B lymphoblastoid cell line and HeLa is an epithelial-like cell line derived from an adenocarcinoma. Daudi cells were grown in RPMI 1640 glutamated supplemented with 10% FCS (Bio-media, Boussens, France), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). HeLa cells were grown in DMEM supplemented as described above. IFN-γ (Roche, Meylan, France) was added to HeLa cells at a concentration of 500 U/ml for 24 h.

Transient transfections experiments and luciferase assays

Five million Daudi cells were electroporated in 0.8 ml of RPMI 1640 medium containing 5 μg of the indicated luciferase reporter construction DNA at 240 V and 960 μF asads with a Bio-Rad electroporator (Gene Pulser; Bio-Rad, France). Immediately after electroporation, samples were resuspended in 5 ml of medium and grown for 24 h. HeLa cells were transfected using the calcium phosphate method without glycerol shock. Six micrograms of plasmid DNA was first mixed with CaCl2 and then with medium containing DNA (1 ml) at room temperature, and added to the cells in 6-well plates for 24 h. Cells were rinsed and incubated with media with or without IFN-γ for 24 h. Cells were harvested, lysed, and the luciferase activity was quantified using a luciferase assay system kit (Promega, Charbonnieres, France) in a TD20 luminometer counter (Promega) following the manufacturer’s procedure. The transcriptional activity of each construction was tested in five independent transfection experiments. Two plasmid preparations from two different clones for each sequence were used and the relative luciferase activities were compared.

EMSA

Nuclear extracts were prepared from the MHC class II-positive Daudi cell line and HeLa cells treated with or without IFN-γ following the procedure described previously (34). Protein/DNA-binding reactions were performed on ice in 20-μl reactions containing 12 μM of HEPES-KOH (pH 7.9), 12% glycerol, 30 mM KCl, 5 mM MgCl2, 0.12 mM EDTA, 0.3 mM DTT, 0.1 μg denatured salmon sperm DNA, 5 μg BSA, and 0.1 μg poly (dI:dC)-poly (dI:dC). Nuclear extracts were preincubated under the above conditions and 50,000 cpm of double-stranded oligonucleotide probe end labeled with [γ-32P]ATP were then added to the reaction and incubated on ice for 30 min. For DNA competition assays, protein extracts were preincubated with competitor DNA for 30 min before addition of the probe. Reactions were then incubated for an additional 30 min.
Supershift experiments were conducted as described previously (34) with a rabbit polyclonal anti-p50 antiserum from Chemicon (Euromedex, Souffelweyersheim, France), various monoclonal anti-p65 Abs from Santa Cruz Biotechnology (Tebu sc-372-G, sc109x and sc109G references; Le Perray-en-Yvelines, France) and Upstate Biotechnology (Euromedex), anti-p65 specific antiserum from the laboratory of J. Imbert (Centre d’Immunologie de Marseille-Luminy), and irrelevant antisera directed against the RFX5 protein. The reactions were loaded on 5% nondenaturing polyacrylamide gels (29:1 acrylamide:bis ratio) in a glycerol-tolerant electrophoresis buffer containing 89 mM Tris, 28.5 mM taurine, and 0.05 mM EDTA. Electrophoresis was conducted for 2 h at 200 V at 4°C. Gels were then fixed, dried, and exposed to x-ray films.

Quantification of HLA-DM and -DR protein expression by Western blot analysis

Total cell lysates were prepared in lysis buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate sodium, 0.1% SDS, and protease inhibitor) from peripheral B cells after positive selection with Dynabeads CD19 (Dynal France, Compiegne, France). Two dilutions of each sample were loaded on 10% SDS-polyacrylamide gels. The proteins were blotted onto Hybond-P membranes (Amersham Pharmacia Biotech, Les Ullis, France) and probed with anti-DMα Ab (5C1Ab kindly provided by P. Creswell, Yale University, New Haven, CT) and anti-β-actin Ab (clone AC-15 from Sigma-Aldrich, St. Quentin, France), followed by HRP-conjugated goat anti-mouse IgG (Immunotech, Marseille, France) and developed using an enhanced chemiluminescence (ECL+Plus) kit (Amersham Pharmacia Biotech). The membranes were stripped and reprobed with an anti-DRα Ab (DA6.147) and anti-β-actin Ab, followed by HRP-conjugated goat anti-mouse IgG and revealed by an ECL+Plus kit. The films were scanned and quantitated using NIH Image software.

Results

Polymorphism in HLA-DM regulatory regions

We sequenced the proximal regulatory regions of the HLA-DM genes from 106 healthy individuals by direct sequencing of the PCR products to characterize both haplotypes and potential heterozygous positions. A nucleotide polymorphism was found in the regulatory regions of these genes. Concerning the HLA-DMA gene, the similarity between the various sequences was found to be higher than 99%. Only two individuals displayed one substitution at position 2292 in regard to the ATG initiation codon (substitution of a guanine by an adenine). This substitution does not map to a consensus regulatory element and is located upstream of the described regulatory motifs. Concerning the HLA-DMB gene, we described two nucleotide variations, one at position 168 and the other one at position 232 in regard to the ATG initiation codon. In both cases, substitutions of a cytosine by a thymine were observed. The substitution at position 168 maps to a putative NF-κB-binding site, and the substitution at position 232 is located between the Y regulatory motif and this NF-κB site. These two substitutions were found to be independent from each other, leading to the description of three HLA-DMB promoter alleles (Fig. 1).

We then sequenced the HLA-DMα and -DMβ regulatory regions of 96 RA patients using a similar approach. We confirmed the

<p>| Table I. Allelic frequencies of the substitutions observed in controls and RA patients |</p>
<table>
<thead>
<tr>
<th>Genes</th>
<th>Dimorphic Site</th>
<th>Allele</th>
<th>Controls (n = 106, frequency %)</th>
<th>RA (n = 96, frequency %)</th>
<th>Fischer’s Exact (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DMA</td>
<td>−292</td>
<td>G</td>
<td>104</td>
<td>93</td>
<td>= 0.67 (NS)</td>
</tr>
<tr>
<td>HLA-DMA</td>
<td>−168</td>
<td>A</td>
<td>2 (0.96%)</td>
<td>3 (1.6%)</td>
<td>= 0.67 (NS)</td>
</tr>
<tr>
<td>HLA-DMA</td>
<td>−232</td>
<td>C</td>
<td>105</td>
<td>92</td>
<td>1 (NS)</td>
</tr>
<tr>
<td>HLA-DMA</td>
<td>−232</td>
<td>T</td>
<td>1 (0.5%)</td>
<td>4 (2.1%)</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 1. Alignment of the HLA-DMA and -DMB regulatory sequences. The consensus motifs: κB, S, J, X1, X2, CAAT, Sp1, and Y motifs are in boxes. The position of the substitution refers to the ATG initiation codon, and the primers sequences are underlined.
same nucleotide substitutions already described in the control population. The allelic frequencies of the substitutions found in the two populations are compared in Table I. For the substitutions observed at positions −292 of the HLA-DMA gene and −168 of the HLA-DMB gene, approximately the same allelic frequencies were found in the two populations (0.96% in controls vs 1.6% in RA and 2.4% in controls vs 2.1% in RA, respectively). The substitution at position −232 of the HLA-DMB gene was found in four RA patients compared with only one healthy individual in >100 tested (allelic frequencies of 0.5% in controls vs 2.1% in RA). A Fisher test was performed to compare these frequencies and the differences between the two populations were found to be statistically not significant (the p values are given in Table I).

Relationship between promoter and coding region polymorphisms

All of the individuals carrying the substitution at position −292 of the DMA gene were HLA-DMA*0101/0102 (Table II). Given that HLA-DMA*0102 allelic frequency is 10% in the general population, the relative overrepresentation of this allele suggests that the HLA-DMA*0102 haplotype carries the substitution. In the same way, the allelic frequency of the HLA-DMB*0103 allele in the population is 18% and was found in all nine individuals presenting the substitution at position −168 of the HLA-DMB gene (Table II). This observation suggests that the HLA-DMB*0103 haplotype carries the substitution at position −168. The HLA-DMB*0101 allele was present in four subjects carrying the substitution at position −232 (Table II). Two of them were homozygous for the coding sequence strengthening the association between this substitution and the HLA-DMB*0101 allele. Thus, the HLA-DMB*0101 gene can be under the control of the wild type (wt) as well as the mutated regulatory sequence. This observation is also true for the other haplotypes described since the allelic frequency of the regulatory region polymorphism is weaker than the one of the coding sequence. These various haplotypes are strongly suggested on the basis of the overrepresentation of certain HLA-DM alleles but we cannot exclude the possibility that various coding sequences can be associated with the substitutions described in the promoter regions. Interestingly, HLA-DMA*0103 that is found more frequently in the RA population (31) is not associated with any of the promoter substitutions. This provides a possible explanation for the absence of association between RA and a particular promoter polymorphism.

Table II. Genotypes of controls and RA patients carrying a polymorphism in the regulatory regions of the HLA-DM genes

<table>
<thead>
<tr>
<th>Allele</th>
<th>A (−292)</th>
<th>T (−168)</th>
<th>T (−232)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes</td>
<td>HLA-DMA</td>
<td>HLA-DMB</td>
<td>HLA-DMB</td>
</tr>
<tr>
<td>Controls</td>
<td>0101/0102</td>
<td>0102/0103</td>
<td>0101/0102</td>
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<tr>
<td></td>
<td>0101/0102</td>
<td>0101/0103</td>
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<td>0101/0103</td>
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<tr>
<td></td>
<td>0101/0103</td>
<td>0101/0103</td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>0101/0102</td>
<td>0101/0103</td>
<td>0101/0101</td>
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<td></td>
<td>0101/0102</td>
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<td>0101/0102</td>
<td>0101/0103</td>
<td>0101/0103</td>
</tr>
<tr>
<td></td>
<td>0101/0103</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* The assignment of one substitution of the regulatory region to one allele of the coding sequence was based on the overrepresentation of this allele (in boldface) in the group of individuals carrying the substitution.

Functional consequences of the polymorphism on promoter activity

It is well established now that mutations in regulatory regions can have important consequences on the transcriptional level of downstream genes. Transient transfection experiments have identified the HLA-DM proximal promoter regions as responsible for their transcriptional activity (20, 21). Thus, the substitutions observed in HLA-DM regulatory regions, and especially the substitution found in the κB site of the HLA-DMB promoter, could potentially induce variation of gene expression. To test the transcriptional activity of the different HLA-DM genes regulatory region sequences, we cloned the various sequences upstream of the luciferase reporter gene and transfected Daudi, IFN-γ-treated, and nontreated HeLa cell lines with the various constructions. We tested two constructions for the DMA regulatory sequences: DMA wt for the sequence found in the database and DMA −292A for the sequence with the G to A substitution at position −292; and three constructions for the DMB regulatory sequences: DMB wt for the sequence found in the database, DMB −168T for the sequence with the C to T substitution at position −168 affecting the putative κB site, and DMB −232T for the regulatory sequence with the C to T substitution at position −232.

Results of the various transfection experiments in Daudi cell line are shown in Fig. 2A. The relative transcriptional activities for the various constructions were compared as a mean from at least five independent experiments. The transcriptional activity of the two DMA regulatory sequences was five times higher than the HLA-DMB ones. The DMA −292A construct displayed the same activity as the empty vector.

**Figure 2.** Functional consequences of the polymorphism of the HLA-DMA and −DMB regulatory regions on transcriptional activity. The description of the various HLA-DM constructs are given in the text. The LTR construct used as a positive control contains the long terminal repeat promoter of HIV-1 upstream of the luciferase gene and the PGL3 construct corresponds to the empty vector. A. Basal transcriptional activity in B cells. Relative luciferase activities are expressed as the mean ± SD (n = 5). B. Relative level of induction by IFN-γ in HeLa cells. Fold inductions were calculated as relative luciferase values of IFN-γ-treated vs untreated cells and are expressed as the mean ± SD of five different experiments.
transcriptional activity as DMA wt. The same result was observed with the DMB –232T and DMB wt constructs. These data suggest that the substitutions at positions –292 of the HLA-DMA and –232 of the HLA-DMB promoters do not have any consequence on the basal transcriptional activity of these genes in vitro. However, the DMB –168T construction showed a 50% decrease in transcriptional activity compared with the DMB wt construct, suggesting that the substitution in the κB site has important functional consequences on the basal transcriptional activity of the HLA-DMB gene in vitro.

The same constructions were transfected in HeLa cells to show a possible influence of the promoter sequence polymorphism on IFN-γ-induced HLA-DM expression. The various constructions tested did not display any basal transcriptional activity in these cells (data not shown). In Fig. 2B, the relative level of induction of the various constructions was compared. HLA-DMB promoter sequences were found to be strongly inducible in IFN-γ-treated HeLa cells, twice more than HLA-DMA regulatory sequences, despite a weaker transcriptional activity in B cells. However, transcriptional activity of DMB –232T and DMB –168T were reduced by 20 and 50%, respectively, when compared with the DMB wt regulatory sequence. On the contrary, the relative IFN-γ induction of the two HLA-DMA regulatory sequences was similar.

Taken together, these results suggest that the constitutive and IFN-γ-induced expression of the HLA-DMB genes could vary according to the polymorphism of their regulatory sequence.

The wild-type and mutated κB sites of the HLA-DMB promoter are functional in vitro

Since the substitution at position –168 of the HLA-DMB regulatory region affects a potential binding site of the NF-κB transcriptional factor family and because of its functional consequences on both basal and inducible transcriptional activities, we investigated by gel shift experiments the in vitro NF-κB-binding ability of the two HLA-DMB κB sites. These two sites, termed DMB-C (wt sequence) and DMB-T (with the substitution), were tested for their ability to inhibit the NF-κB-binding activity obtained from B-EBV nuclear extracts to the functional κB site of the IL-2 receptor α-chain gene (κB IL-2Rαwt) (Fig. 3A) (35). An excess of the unlabeled DMB-C or DMB-T oligonucleotides (50–200 times) was able to compete the protein binding to the κB IL-2Rαwt probe. This binding inhibition was as efficient as the competition with the unlabeled κB IL-2Rαmut oligonucleotide. The specificity of this competition was confirmed by showing that a nonspecific competitor or a mutated κB sequence (κB IL-2Rαmut), containing three mutations disrupting the binding site, was not able to compete the NF-κB binding. These experiments strongly suggest that the wt and mutated κB sites of the HLA-DMB regulatory regions are both functional in vitro.

To characterize and compare the protein factors bound to the different κB sites, we labeled the κB IL-2Rαwt site (Fig. 3B, lanes 1–3) and the two DMB sequences (DMB-C lanes 4–6 and DMB-T lanes 7–9), and we performed supershift experiments with anti-p50- and -p65-specific Abs. We tested DNA-protein binding with nuclear extracts from Daudi as well as IFN-γ-treated and non-treated HeLa cell lines. The binding profiles of the two DMB probes were exactly the same for all nuclear extracts tested. A representative binding profile obtained with nuclear extracts from the Daudi cell line is shown in Fig. 3B. A supershift of the protein-DNA binding was observed in the presence of the anti-p50 Ab (Fig. 3B, lane 2 for κB the IL-2Rαwt probe and lanes 6 and 9 for the DMB-C and DMB-T probes, respectively). Under experimental conditions used, no protein-DNA complex was supershifted in the presence of the various p65 Abs. A representative result is shown in Fig. 3B (lanes 3, 7, and 10). The addition of an irrelevant antiserum directed against the RFX5 protein was also tested as negative control and did not result in a supershifted complex (lane 4, Fig. 3B). Thus, carrying or not the substitution at position –168, the HLA-DMB κB sites seem to have the same protein-binding ability in vitro. The differences in transcriptional activity observed in transfection experiments could not be related to differences in the NF-κB-binding ability of the HLA-DMB κB sites.

Quantification of HLA-DM and DR transcripts in controls and RA patients

To investigate the variability of HLA-DM expression, as well as to compare the level of expression in controls and RA patients, we performed semiquantitative RT-PCR analysis on HLA-DM transcripts from peripheral B lymphocytes isolated from 7 healthy individuals and 10 RA patients. The polymorphism of the regulatory regions of the HLA-DM genes from all individuals was determined by sequencing. Only one RA patient displays a polymorphism in the regulatory region of the HLA-DMB gene (substitution at position –232). The quantification of the HLA-DM transcripts was normalized using the GAPDH transcripts and the HLA-DM: GAPDH ratios were compared for the various individuals (Fig.
4). First, we observed similar levels of HLA-DMA and -DMB transcripts within each population with a weak variability leading to a homogenous group of values. Mean values of the HLA-DMA:GAPDH ratio were 1.4 ± 0.2 for controls (n = 3) and 0.5 ± 0.2 for RA patients (n = 5). We compared the mean values using the unpaired t test, and the difference between controls and RA appeared to be extremely significant (p = 0.0007). Concerning the HLA-DMB:GAPDH ratio, the means values were 1.3 ± 0.2 for controls (n = 7) and 0.5 ± 0.2 for RA patients (n = 10). The difference between RA and controls was also extremely significant (p < 0.0001). To determine the specificity of the results observed for RA, a third group containing five patients suffering from non-autoimmune inflammatory rheumatism (ankylosing spondylitis, AS) was included in this study. Mean value of the HLA-DMA:GAPDH ratio for AS patients was 1 ± 0.2 (n = 4). The difference between controls and AS was not significant (p = 0.0632), whereas the difference between RA and AS was considered very significant (p = 0.0021). Mean value of the HLA-DMB:GAPDH ratio for AS patients was 1 ± 0.2 (n = 5). The difference between controls and AS patients was not significant (p = 0.0562), in contrast to the difference between RA and AS patients that was extremely significant (p = 0.0002).

These results suggest that HLA-DMA and -DMB transcript expression in the RA population is three times less than in controls, suggesting a down-regulation in this autoimmune disease. The specificity of this underexpression for RA patients is supported by a normal expression found in the AS group.

HLA class II genes are usually known to be coordinately expressed. To see whether the HLA-DR genes were also down-regulated, we investigated the HLA-DRB expression using quantitative RT-PCR as previously described (33). The quantification of the HLA-DRB transcripts was normalized to the GAPDH transcripts and the HLA-DRB:GAPDH ratios were compared for the various individuals (Fig. 4B). Mean values of the HLA-DRB:GAPDH ratios were 0.5 ± 0.06 (n = 6) for controls and 0.6 ± 0.2 for RA patients (n = 10). The relative HLA-DRB expression was found to be similar between RA and controls; however, the SD of the values was higher in the RA population.

An arbitrary DM:DR ratio was calculated for the two populations as illustrated in Fig. 4C. This ratio was found to be three times higher in controls than in RA patients (2.8 ± 0.5 for controls vs 1 ± 0.6 for RA). As the HLA-DRB expression is similar in the two populations, the difference of the DM:DR ratios observed reflects the HLA-DM down-regulation in RA patients. This difference was considered to be extremely significant using the unpaired t test (p < 0.0001). These results strongly suggested a specific HLA-DM down-regulation in RA patients.

Quantification of HLA-DM and DR protein levels in controls and RA patients

The expression of the HLA-DM molecules has been reported to be directly correlated to the level of the transcripts (16). To investigate the variability of the HLA-DM protein levels in RA patients, we quantified the DMAP protein by Western blot analysis of total cell lysate from B lymphocytes isolated from 12 RA and 10 controls. The blots were probed with anti-DMA and anti-β-actin Abs (Fig. 5A), stripped, and reprobed with anti-DRα and anti-β-actin Abs (Fig. 5B). The β-actin expression was detected to check the relative protein quantity loaded on the gel and to compare the HLA-DMA and -DRα protein levels between the various individuals. Because of the short linearity of the signal emission in Western blot experiments, we cannot normalize the DMAP and DRα expression to β-actin for all of the individuals tested on the various gels. Thus, we performed numerous experiments to gather individual results obtained with the same hybridization and revelation conditions. Gels representative of all of the results obtained for HLA-DMA and -DRα protein expression are shown in Fig. 5. In Fig. 5A, the two gels showed clearly a down-expression of the DMAP protein in RA patients. Intensities of the signal were quantitated using NIH image software and the ratio between DMAP and β-actin were compared for the various individuals (Table III). Considering the data obtained from the various gels in the range of signal linearity, we detected a 1.5- to 4-fold lower DMAP expression in RA patients compared with controls. The DRα protein expression was found to be slightly affected or unchanged in RA patients. Indeed, some of the RA patients showed no variation of DR expression (Fig. 5B and Table III: compare a/b to c/d and also j to l), whereas others displayed a weaker (compare j to k and also e/f to g/h) or higher (compare i to k/l) DRα expression.

As expected, the DR and DM protein expression levels are directly correlated to their respective transcript expression. The HLA-DM down-regulation was then found at both transcriptional and protein levels in RA patients.

![FIGURE 4. Down-regulation of HLA-DM transcripts in RA. A. Quantification of HLA-DMA and -DMB transcripts by semiquantitative RT-PCR. The ratios of fluorescence intensities for DM and GAPDH transcripts are indicated with means and SD in each group (see text for details). The differences of the mean values were compared according to the unpaired t test and the p values are indicated. B. Quantification of HLA-DRB transcripts by competitive RT-PCR in controls and RA patients. The ratios between DRB and GAPDH cDNAs are represented with means and SD in each group. C. Calculated DMB:DRB ratios.](http://www.jimmunol.org/)
FIGURE 5. Down-regulation of HLA-DM protein expression in RA. A. Comparison of the HLA-DM protein expression between controls (C) and RA patients (RA). Two dilutions of protein samples were loaded on the gels (labeled 1:2 and 1:1). The protein was revealed by an anti-DM Ab (DA6.174) and an anti-β-actin Ab to check the relative protein quantity loaded on the gels. The two gels shown are representative of the results obtained for all controls and RA patients tested. B. Comparison of the DRα protein expression between controls and RA patients. The same gels were reprobed with an anti-DRα Ab (DA6.174) and the same anti-β-actin Ab. Three gels representative of the results obtained for all individuals are shown. One dilution of the samples is represented and the individuals are labeled (a–l). The same individuals (a–g) are shown in A and B.

Discussion

HLA-DM is able to affect the peptide repertoire bound to HLA class II molecules mainly by two ways: 1) HLA-DM acts as a peptide editor, selecting high-intrinsic kinetic stability class II-peptide complexes for presentation on the cell surface, and (2) HLA-DM keeps empty class II-binding grooves suitable for peptide loading at low lysosomal pH even when a low amount of peptide is available (36). It is reasonable to assume that the efficiency of the editing process is dependent on numerous factors, such as the amount of foreign or self peptide available for peptide loading, the ratio of high- vs low-intrinsic kinetic stability class II-peptide complexes, the pH, the relative amount of HLA-DM in the respective compartment of APC compared with the total amount of class II molecules, the residence time of class II molecules in HLA-DM-positive compartments, and also the HLA-DR allele (18). The action of the HLA-DM molecule is dose dependent (37), suggesting that a variation of the HLA-DM gene expression would have important consequences on the peptide repertoire bound to HLA class II molecules and presented to CD4⁺ T cells (19).

We described in this study for the first time a nucleotide polymorphism in the regulatory regions of the HLA-DM genes. One substitution was observed 292 bp upstream of the ATG initiation codon in the regulatory region of the HLA-DM gene, and two substitutions were found in the HLA-DMB regulatory regions (at positions −168 and −232 in regard to the ATG initiation codon), leading to the description of respectively two and three different regulatory sequences for the HLA-DM and -DMB genes. This polymorphism has a frequency of <3% in the general population. It seems to be preferentially associated with certain HLA-DM alleles. To entirely eliminate a possible influence of linkage disequilibrium between alleles at the DM locus and RA-associated DRB1 alleles, the control population was chosen as a group of HLA-DRB1-matched controls. The allelic frequencies of the described alleles are not statistically different between RA and control populations.

We also investigated the in vitro functional consequences of the nucleotide substitutions described on the transcriptional activity using a reporter system assay. The polymorphism of the HLA-DMA regulatory region did not affect the basal transcriptional activity in the B-EBV lymphoblastoid cell line nor the IFN-γ induction in HeLa cell lines. By contrast, the substitution in the HLA-DMB regulatory region at position −232 was found to be responsible for a 20% decrease in the IFN-γ induction in HeLa-treated cells, without affecting the basal transcriptional activity in the Daudi cell line. Furthermore, the substitution at position −168 of the HLA-DMB regulatory region, located in a putative NF-κB-binding site, was found to be responsible for a 50% decrease in transcriptional activity in both conditions (basal and IFN-γ induction). To further analyze the consequence of this substitution, we evaluated the in vitro protein-binding ability of the HLA-DMB wt vs mutated κB sites. These two κB sites were found to be functional in vitro, with similar binding ability for all nuclear extracts tested (Daudi, IFN-γ-treated or nontreated HeLa cell lines). The differences in transcriptional activity observed in transfection experiments could not be explained by the NF-κB-binding properties of the different HLA-DMB κB sites. Nevertheless, the polymorphism found in the proximal regulatory regions of the HLA-DMB genes is responsible for variations in transcriptional activity and IFN-γ inducibility in vitro and could be responsible for variation of HLA-DM gene expression in vivo.

Table III. Intensities of the signal obtained in Western blot experiments shown in Fig. 5a

<table>
<thead>
<tr>
<th>Samples</th>
<th>Dilution</th>
<th>DMA</th>
<th>β-actin</th>
<th>DMA:β-actin</th>
<th>Sample</th>
<th>Dilution</th>
<th>DRA</th>
<th>β-actin</th>
<th>DRA:β-actin</th>
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<td>170</td>
<td>1.9</td>
<td>a</td>
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<td>300</td>
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<td>270</td>
<td>2.2</td>
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<td>400</td>
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*The quantification of the signal was done using NIH Image software. The ratio between DMA:β-actin and DRA:β-actin was calculated. RA samples are in boldface characters.
We evaluated the variability of HLA-DM and -DR expression in control and RA populations by semiquantitative or competitive RT-PCR as well as Western blot experiments. We observed similar levels of HLA-DMA and -DMB transcripts within each population, with a weak variability for each group of values. In the RA population, we observed an underexpression of HLA-DM with a highly significant difference between the RA and control population. This underexpression seems to be specific for RA patients, or at least an autoimmune situation, since the HLA-DM transcript expression appeared to be normal in the inflammatory arthritis group. The down-regulation observed could not be the consequence of DM promoter polymorphism since only one RA patient carries a substitution at position -232 in the HLA-DMB regulatory region. This substitution does not affect the HLA-DMB transcriptional activity in vitro as it is demonstrated in the first part of this study. HLA-DM is specifically down-regulated in RA since the HLA-DR expression appeared to be not or slightly affected. An arbitrary DM:DR ratio was found to be three times higher in controls than in RA patients, confirming the specific DM down-regulation in RA patients. We investigated the HLA-DR and -DM protein expression and confirmed the DM down-regulation at the protein level. These data suggest that disease specific transcriptional or posttranscriptional regulatory mechanisms are involved in HLA-DM gene expression in RA.

To see whether the reduced expression of DM molecules could be due to the treatment, we examine drug regimens of the RA patients. All of the patients studied received either various treatments (methotrexate, cyclosporine, sulfalazine, corticoids, hydroxychloroquine) or no treatment (one patient in remission), suggesting that the underexpression observed is not a consequence of the treatment. This argument is supported by the fact that similar treatments (sulfalazine and corticoid) were given to the four AS patients without any consequence on the HLA-DM expression. Another explanation for this down-regulation could be the activation state of the peripheral B cells from RA patients. To rule out this hypothesis, the expression of B cell activation markers (CD23, CD5, CD80, CD86, CD69, CD71, CD95, and CD25) was compared between controls and RA patients. An increase of CD25-positive B cells and a decrease of CD5-positive B cells were found in RA patients with active disease compared with controls (data not shown). No differences in the expression of CD23 and other activation markers were found between controls and patients with nonactive disease, although all patients displayed a low expression of HLA-DM molecules.

Even if HLA-DM genes are usually considered to be coexpressed and coregulated with classical HLA class II genes, this study demonstrated a specific underexpression of HLA-DM transcripts and protein in RA. The non-coordinate regulation of HLA-DM and -DR genes has been previously described in a mutant cell line (38) and suggests that the basic coregulation within the classical class II genes, some mechanisms could be more specific for HLA-DM genes. HLA-DM and -DR molecules are localized in the class II peptide-loading compartment in a 1:5 ratio (15, 39). The down-regulation of the HLA-DM molecule observed in RA could be responsible for a decreased amount and function of HLA-DM in this compartment. If the down-regulation observed is restricted to the periphery, the diversity of the peptide repertoire presented by HLA class II molecules would be broader than in the thymus, leading to the potential activation of autoreactive T cell clones in periphery that would not have been deleted during the negative selection. This hypothesis agrees with the description of a higher number of autoreactive T cell clones in RA (40).

The modification of the MHC class II-bound peptides could be monitored by several approaches. A widely used parameter to determine the intrinsic kinetic stability of the MHC class II-peptide complex is to study the stability of the complex in SDS (41). Another possibility would be to investigate the level of expression of the class II-CLIP complexes at the cell surface; however, the DRB1*0401 molecule associated with RA has been demonstrated to release CLIP independently of the DM molecule. The last approach will be the elution of the peptide-bound HLA class II molecule with the comparison of the peptide profile according to the level of HLA-DM expression.

Beside the role of HLA-DM as peptide editor, this molecule is able to keep empty class II-binding grooves in a suitable conformation for peptide loading at low lysosomal pH. The HLA-DRB1*0401 molecule, associated with RA, has a low-intrinsic stability and thus exhibits a high dependency on HLA-DM to prevent its aggregation and denaturation (15). In the absence of HLA-DM, beside the possibility that the class II molecules would be stabilized by low-kinetic stability peptides, it is possible that the DRB1*0401 molecule would be chaperoned by other molecules like heat shock protein. This hypothesis has already been suggested after the demonstration that the DRB1*0401 molecule interacts with the constitutive 70-kDa heat shock protein, directly targeting the HLA class II molecules to lysosomes (42). Recently, the involvement of the heat shock cognate protein 73 in MHC class II Ag presentation had been confirmed by another group (43).

In conclusion, we demonstrated a polymorphism of the regulatory regions of the HLA-DM genes with functional consequences on their transcriptional activities, and our present work brings evidence for a specific down-regulation of HLA-DM RNA and protein expression in RA. The molecular basis of this down-regulation is unknown and even if it is just a secondary event due to autoimmune and/or other processes, this underexpression, observed in the peripheral blood B cells from RA patients, may have important consequences on the Ag presentation.

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References


