HLA-DP molecules bind cobalt: a possible explanation for the genetic association with hard metal disease

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Metal dust inhalation induces an interstitial lung disease which may progress to pulmonary fibrosis (hard metal disease, HMD). Cobalt is believed to be the pathogenic agent of HMD. A strong genetic association of HMD with some HLA-DP alleles has been reported although the role of these molecules in the occurrence of the fibrotic disorder remains unclear. A possible explanation of these findings is that HLA-DP but not other HLA class II molecules can bind cobalt. This could have as a consequence an HLA-DP-mediated specific activation of the immune system. To test this hypothesis, we have set up an *in vitro* binding assay using ^{57}Co and purified HLA-DP and -DR molecules. The results indicate that HLA-DP but not HLA-DR molecules bind cobalt. Moreover, the presence of HLA-DP Glu β69, which is associated with susceptibility to HMD, determines a higher metal uptake. Molecular modelling of HLA-DP2 molecules places the Glu β69 residue in a position relevant in determining peptide specificity. The possibility that binding of cobalt by HLA-DP molecules can interfere with their antigen presenting functions is discussed.

Key words: HLA-DP / Cobalt / Fibrosis

1 Introduction

A first report describing a pulmonary disorder associated with occupational exposure to hard metal dusts was published in 1940 [1]. Since then, a syndrome named "hard metal disease" (HMD) has been characterized in detail and comprises a wide range of clinical signs and symptoms [2–4]. Since HMD is induced by inhalation of cobalt ions, sintered or not with other hard metals, cobalt is considered the main triggering agent [5–7]. Exposure to cobalt dust may produce several respiratory disorders, including upper respiratory tract irritation, asthma, and the interstitial lung disorder which may progress to the pulmonary fibrosis. An important hallmark of HMD is the presence of multinucleate giant cells in the bronchoalveolar lavage, underlying the occurrence of an inflammatory process in the lung tissue [8, 9]. Although the clinical aspects of the disease are well defined,

the pathogenesis remains unclear. The possible involvement of immunological mechanisms has been pointed out by the finding of a strong association of HMD with HLA-DP allelic products sharing a Glu residue at position β69 [10]. Interestingly, the same association was found in chronic beryllium disease (CBD), suggesting a common pathogenic pathway which might involve a wider range of divalent ions [11]. Accordingly, it has been hypothesized that some metal ions can bind directly HLA-DP molecules and then be presented to the T cells. To explore this possibility, we have performed a direct binding test by using ^{57}CoCl$_2$ and purified HLA-DP and -DR molecules and shown that only HLA-DP molecules bind cobalt in a pattern that appears to correlate with susceptibility to HMD [10].

2 Results

2.1 HLA-DP but not -DR molecules bind ^{57}CoCl$_2$

In a previous study, among the HMD-affected individuals, the HLA-DPB1*0201 (Glu$^+$ β69) allele was found to be increased, whereas -DPB1*0401 (Lys$^+$ β69), which is the most common allele in the Caucasoid population, was found to be decreased as compared with an

exposed, healthy population [10]. To investigate whether HLA-DP molecules can bind cobalt, an *in vitro* system was established using $^{57}CoCl_2$. The method was set up first using different concentrations of BSA, which is a good binder of divalent cations (not shown). The two HLA-DP molecules, *0201 from VBP and *0401 from Co1 cell lines, were then purified and tested in a direct metal binding assay. Concomitantly, HLA-DR molecules from the same two cell lines were also purified and underwent the same procedure. MHC purity was controlled by estimating the protein yield, under denaturing and non-denaturing conditions, on SDS-PAGE performed in parallel to Western blot analysis (data not shown). For each protein preparation, a small volume was iodinated and aliquots corresponding to the same trichloroacetic acid (TCA)-precipitated cpm were used for the binding tests. Fig. 1a shows the densitometric analysis of a representative experiment using this cobalt binding assay.

HLA-DP*0201 molecules bound $^{57}CoCl_2$ at least three times more efficiently than HLA-DP*0401 molecules. The presence of unlabeled $CoCl_2$ or EDTA hindered HLA-DP*0201 binding. That an equivalent amount of protein preparations was used for each sample was controlled by chemiluminescence as described in Sect. 4 (Fig. 1b). Under the same experimental conditions HLA-DR molecules bound $^{57}CoCl_2$ at a very low level. Interestingly, in a previous report it has been shown that Zn^{2+}, a Co^{2+} equivalent ion, has no binding site in HLA-DR1 molecules [12].

2.2 Glu β69 correlates with a higher cobalt binding

The HLA-DPB1 gene comprises six hypervariable coding regions, denoted as A, B, C, D, E and F. HLA-DPB1*0201 and DPB1*0401 alleles differ by three of these regions: the conservative substitution Val to Ala at position 36 (B); Asp Glu to Ala Ala at positions 55 and 56 (C); and Glu to Lys at position 69 (D) [13]. Glu as well as Asp are known to be good ligands for Co^{2+} ions [14]. Thus, in principle, the difference in cobalt binding between DP*0201 and DP*0401 molecules might correlate with either of these two residue substitutions, C or D. The relevance of Glu β69 was analyzed testing cobalt binding to DP*0402 molecules that differ from HLA-DP*0201 only at this position (Lys instead of Glu). To verify both the state and the amount of the protein preparations used, PAGE of the three (HLA-DP*0201, *0401, *0402) molecules was run under non-denaturing conditions (Fig. 2). A major comparable band is visible at the position expected for the heterodimer in the three samples. The stronger signal present at the origin of the gel represents complexed molecules, an expected feature under the non-denaturing gel electrophoresis conditions
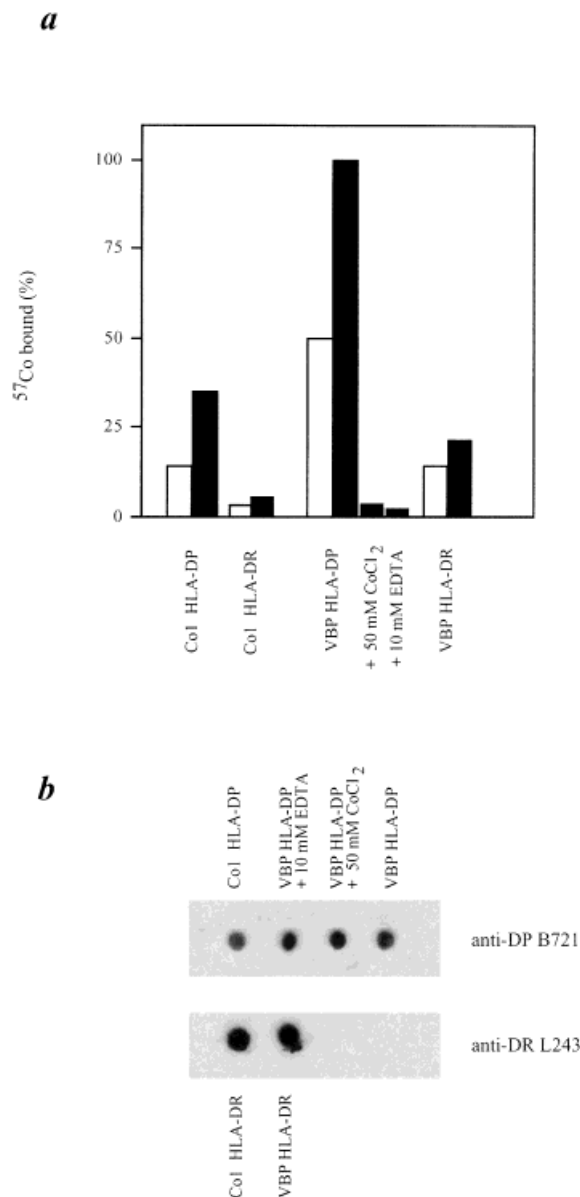

Figure 1. Densitometric analysis of $^{57}CoCl_2$ binding of HLA-DP and HLA-DR molecules. Protein amounts were normalized by radiolabeling aliquots of purified HLA class II molecules and binding was performed at serial twofold dilutions: 1×10^6 ^{125}I cpm (open bars) and 3×10^6 ^{125}I cpm (solid bars). (a) shows the PhosphoImager densitometric measurements expressed as percentage of the density given by the 3×10^6 ^{125}I cpm equivalent of DP*0201 molecules from the VBP cell line. Cobalt binding was hindered by the presence of 50 mM unlabeled $CoCl_2$ or of 10 mM EDTA (pH 8). (b) shows the equivalent amount of proteins (corresponding to 3×10^6 ^{125}I cpm) spotted on nitrocellulose membrane and treated as above but without $^{57}CoCl_2$ and processed for chemiluminescence detection of proteins as described in Sect. 4.4.
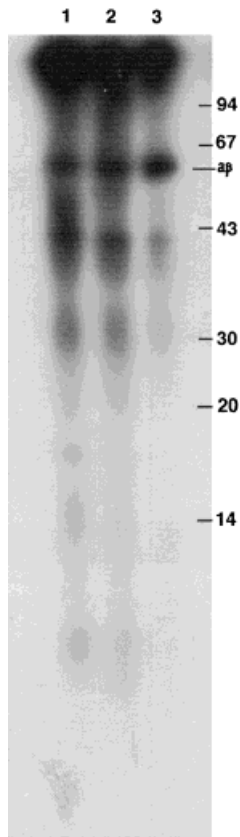
Figure 2. Gel electrophoresis of TCA-precipitated HLA-DP molecules. Immunopurified HLA-DP*0201 (lane 1), *0401 (lane 2) and *0402 (lane 3) molecules were iodine labeled, precipitated and 4×10^6 cpm were run in each lane in a 12.5 % polyacrylamide gel under non-denaturing conditions. Positions of molecular mass markers phosphorylase B (94 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa) are indicated at the right.

used here and verified in this particular case by Western blot (not shown). Equivalent amounts of non-labeled preparations were mixed with ^{57}CoCl$_2$. Interestingly, HLA-DP*0402 molecules bound less ^{57}CoCl$_2$ than HLA-DP*0201, thus pointing to Glu β69 as a cobalt ligand (Fig. 3a). However, the difference observed between HLA-DP*0402 and HLA-DP*0401 subtypes suggests that also Asp β55 and/or Glu β56 (Ala Ala in HLA-DP*0401 molecules) are cobalt ligands contributing one or two additional binding sites. Therefore seems to be there a correlation between the intensity of cobalt uptake and the number of acidic residues in the DPβ1 domain (Fig. 3b).
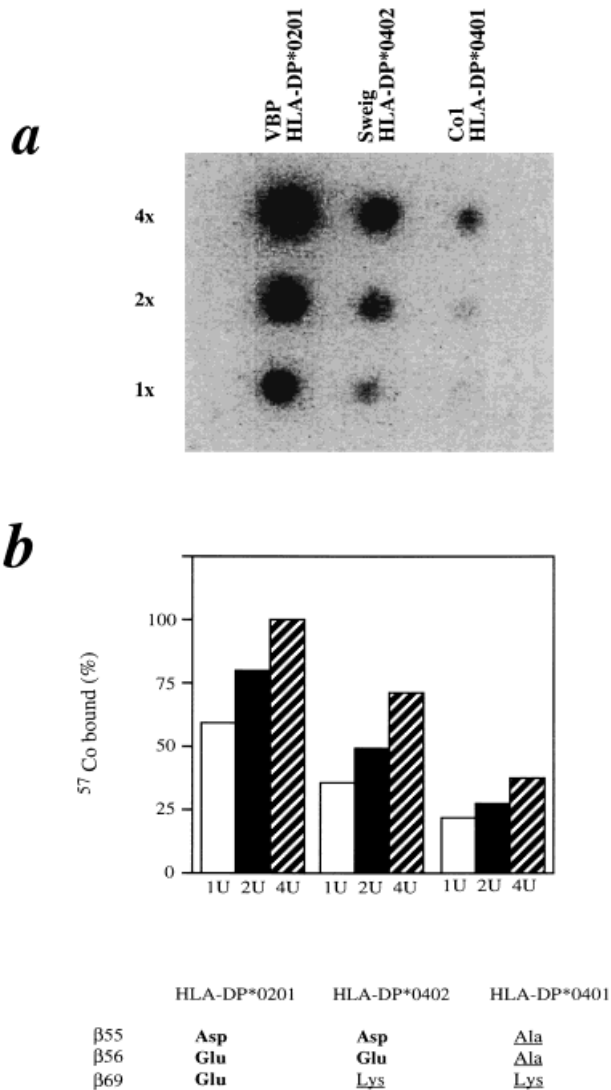
Figure 3. Differential ^{57}CoCl$_2$ binding of HLA-DP alleles. The following B cell lines were used: VBP (HLA-DPB1*0201/*0201), Sweig (HLA-DPB1*0402/*0402), Co1 (HLA-DPB1*0401/*0401). *In vitro* ^{57}CoCl$_2$ binding was performed under the same conditions as in Fig. 1, being defined in this case as 1.8×10^6 ^{125}I TCA-precipitated cpm. The autoradiographic image is shown in (**a**) and the corresponding densitometric value in (**b**) is expressed as a percentage of the density given by 4U ($= 7.4 \times 10^6$ ^{125}I cpm) of HLA-DP*0201 molecules.

2.3 Localization of the putative cobalt-binding sites by molecular modeling

Structural and functional studies on HLA-DP molecules have been hampered by the low expression of these molecules on the cell surface and no crystallographic image is available yet. However, HLA-DP2 can be modeled on the HLA-DR1 structure and the location of most residues in the DP α- und β-chains can be predicted. The

alignment of -DR and -DP β chains implied a two-amino acid shift starting approximately from position β22, whereas α chains can be aligned with no amino acid shift. Based on the modeling (Fig. 4), DP β69 corresponds to the DR β71, a P4 pocket residue that has been shown to contribute to the specificity of bound peptides [15, 16]. The side chains of DP2 Glu β69 and DP2 Asp β55 are projected towards the peptide-binding cleft, although they are located in non-contiguous parts of the molecule. DP2 Asp β55 is predicted to be localized in the P9 contact region [17]. It is in the proximity of the conserved residue Arg α76 and, similarly to what has been observed in the DR1 structure (Asp β57/Arg α76), it might form a salt bridge underneath the bound peptide. The DP2 Glu β56 side chain is oriented outside the peptide-binding groove in the proximity of the nonpolymorphic residue Glu β57 forming a negatively charged region external to the molecules. It is unlikely that this acidic site is involved in peptide selection.

3 Discussion

The mechanisms by which metals induce allergic or autoimmune-like reactions are still obscure. The findings that some metal-related diseases are strongly associated with the HLA-DPB1 locus, whereas some others show no HLA association, suggest that several mechanisms may operate. In this respect, cobalt is particularly interesting since it can induce different pathologies: in the case of metal-specific contact hypersensitivities (CHS), cobalt sensitivity is observed in a high percentage of nickel-sensitive individuals and, in such case, no convincing genetic association has ever been reported. On the contrary, a strong genetic association with some HLA-DP alleles paralleling what has been described in the case of CBD [11] has been reported for HMD, suggesting that CHS and HMD originate from different molecular mechanisms.

Several studies have described T cells specific for metals like nickel [18], gold [19], beryllium [20] and cobalt [21], indicating that metal ions can interfere with antigen presentation [22–23].

In the case of nickel-induced allergy, the metal was found to inhibit the reactivity of an antigen-specific T cell clone probably by interacting with the loaded peptide [24]. Given the promiscuity of the HLA class II molecules [25, 26], this could explain why those allergies show little or no HLA association [27–29]. HLA-DP molecules bind peptides and they are similar in length and properties to
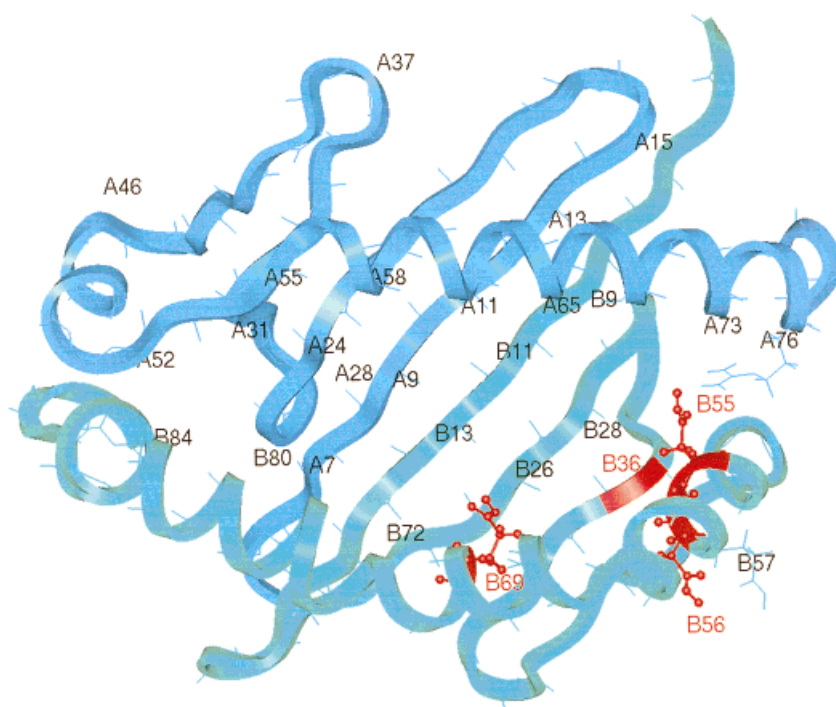
Figure 4. Molecular model of HLA-DP*0201 peptide-binding groove. The protein backbones are overlaid by ribbon representation, the blue parts belong to the α chain and the turquoise parts to the β chain, respectively. Sequence positions of some amino acids are indicated, where 'A' refers to the α chain, 'B' to the β chain, and number 1 is assigned to the amino acid C-terminal of the putative signal peptide. Amino acids β 36, 55, 56 and 69 are shown in red. The relevant amino acids (β 55, 56 and 69) are furthermore represented as "ball and stick" models.

those isolated from HLA-DR and -DQ [30]. The strong association of HMD with HLA-DP Glu β69, the experimental observation that cobalt-binding correlates with the number of acidic residues in the HLA-DP groove and the lack of an appreciable binding by HLA-DR all indicate the existence of an alternative pathogenic mechanism by which the direct interaction between cobald and HLA-DP is part of the disease process.

Some features of the HLA-DP-cobalt interaction can be inferred by molecular modeling. Since the more common Co^{2+} coordination geometry is tetrahedral with an acceptable distance from ligands of approximately 2Å [30–32], it is evident in the model (Fig. 4) that, when the three polymorphic residues Asp β55, Glu β56 and Glu β69 are present, *i. e.* in the DP2 molecule, they are too far away to take part in the same $^{57}Co^{2+}$ binding site. The data suggest instead that there is more than one independent metal binding region in the molecule and Glu β69 is likely to contribute one of them. In HLA-DR1 molecules Asp β57 (homologous to HLA-DP2 β55) is engaged in a salt bridge with the facing Arg α76 and it is resonable to assume that it also occurs between HLA-DP2 Asp β55-Arg α76 facing residues. Since HLA-DR cobalt binding is negligible, it is unlikely that in HLA-DP2 molecules the homologous region is determinant for the metal binding.

Glu β56, possibly in cooperation with the closely located Glu β57 which is conserved in all DP molecules, might also contribute to an alternative Co^{2+} pocket, but external to the peptide-binding cleft. Assuming that the HLA class II-peptide-TCR complexes are similar to those formed by HLA class I [34–36], this metal pocket is unlikely to affect the interaction with the TCR. The position Glu β69 is predicted to be placed within the groove and it probably contributes to the P4 contact region. Moreover, *in vitro* binding tests indicate that this residue is responsible for the differences observed between HLA DP*0402 and HLA DP*0201. Therefore Glu β69 appears to be critical for both Co^{2+} binding and peptide presentation.

Once cobalt is bound to HLA-DP molecules, it might induce structural changes of the peptide-MHC complex, but it is also possible that cobalt is required to load a new set of peptides. Cobalt could bind empty HLA-DP molecules, thus producing a charge modification of the P4 pocket, where the Glu β69 residue is located. As a consequence, previously ignored tissue-specific self peptides, to which patients have not been made tolerant, can be loaded. In both cases, a specific T cell response might occur which may trigger the tissue distruction preceding fibrosis.

In conclusion, Glu β69, although it is not the only ligand for cobalt in the HLA-DP molecule, is likely to be itself a key factor in the pathogenesis of metal-driven autoimmune diseases such as HMD [37, 38].

4 Materials and methods

4.1 Cell lines

HLA-DP-homozygous cell lines were used. Sweig (HLA-DRB1*1101/*1101; HLA-DPB1*0402/*0402) was derived from the 10th Histocompatibility Workshop. Co1 and VBP are two EBV-transformed B cell lines generated in our laboratory from PBL of two healthy donors. The cell lines were HLA-class II typed by genomic DNA typing [39, 40] and were HLA-DRB1*1303/*0101; HLA-DPB1*0401/*0401 (Co1) and HLA-DRB1*0401/*0701; HLA-DPB1*0201/*0201 (VBP).

4.2 HLA class II purification

HLA-DP and -DR proteins were immunoprecipitated from B cell line batches as described by Gorga et al. [17] with minor modifications. Briefly, 300 mg of EBV-transformed human B cell lines were resuspended in a freshly prepared lysis buffer containing 50 mM Tris pH 7.5, 1 % NP40 (BDH, GB), 0.15 M NaCl and complete EDTA-free protease inhibitor cocktail tablets (Boehringer Mannheim). Cell lysates were precleared by overnight incubation at 4 °C with 50 µl packed protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden). Immunoprecipitations were performed in lysis buffer with anti-DR L243-Sepharose, followed by anti-DP B7/21-Sepharose beads. Each incubation step was for 2 h at 4 °C. Beads were washed extensively and elution was carried out in 50 mM diethylamine, 10 % glycerol, 0.1 % NP40, pH 11.5. Immunoprecipitates were concentrated using Microcon 30 (Amicon) and resuspended in 100 µl 20 mM Hepes pH 7.2, 0.85 % NaCl buffer. The purity of MHC preparation was controlled loading biotinylated proteins on SDS-PAGE under reducing and non-reducing conditions followed by Western blot analysis (Hybond-ECL membrane, Amersham). Protein detection was carried out using peroxidase-streptavidin [Chemiluminescence Blotting Kit (biotin/streptavidin), Boehringer Mannheim].

4.3 Iodination

Aliquots of the immunoprecipitated HLA-DR and -DP molecules were radioiodinated in 200 µl PBS with 30 µCi ^{125}I (Amersham) and 1 mg Iodo-Gen (Pierce) [41]. Samples were TCA precipitated, using 5 µg BSA (Sigma) as carrier, and assayed in a γ-counter. Volumes were adjusted to the same protein concentration on the basis of TCA-precipitable cpm.

Aliquots of labeled proteins were precipitated in the presence of BSA with a mixture of 50 % ethanol/25 % acetone/

25 % methanol for 1 h at -80 °C, centrifuged for 20 min in an Eppendorf centrifuge at 14 000 rpm, dried and resuspended in 20 μl non-reducing SDS sample buffer. Of each sample, 2 μl were counted in a γ-counter and the equivalent of 4×10^6 cpm was loaded and run on an SDS-12.5 % polyacrylamide gel. Gels were dried and autoradiographed.

4.4 Cobalt binding assay

Purified HLA class II proteins, HLA-DP*0201, *0401 and *0402 and HLA-DR, adjusted to the same concentration and resuspended in 200 mM MOPS pH 5.1, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 2 mM CHAPS, were mixed with 0.5 μCi $^{57}CoCl_2$ (Amersham; 6.2 mCi/μg) in a final volume of 50 μl. Acidic conditions (pH 5.1) were used to mimic the HLA-class II peptide loading microenvironment [42–44]. After 1 h incubation at 37 °C, the samples were passed through a 0.2-μm nitrocellulose transfer membrane (Protran Nitrocellulose Membrane, Schleicher and Schuell, Germany) with the MOPS buffer [45]. Under these conditions, only protein-bound $^{57}CoCl_2$ is retained by the membrane. After extensively washing with the binding buffer, filters were subjected to autoradiography on a phosphoimager. A corresponding amount of proteins was spotted on a nitrocellulose membrane and incubated with specific anti-HLA class II mAb (B7/21 and L243) [46–47]. After three washes, horseradish peroxidase-labeled secondary antibody conjugate (Amersham) was added for 30 min. Chemiluminescence was carried out by using BM chemiluminescence blotting substrate (POD) from Boehringer Mannheim following the manufacturer's instructions. The membranes were exposed to film for 60 s at room temperature.

4.5 Molecular modeling of HLA-DP*0201

Sequence and domain structure information were obtained from the Swiss-Prot database. Sequences corresponding to the extracellular parts of α and β chain, respectively, were individually submitted to the Swiss-Model Protein Modelling Server (swissmod@ggr.co.uk) via the "first approach mode". Both resulting models were subsequently resubmitted via the "combine mode", with 1DLH (3D structure of HLA-DR1) as a template [48–50]. The resulting 3D model of the extracellular domain of HLA-DP2 was processed for representation by InsightII (MSI, San Diego, CA).

5 References

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