HLA-DRB1*0401 and HLA-DRB1*0408 Are Strongly Associated with the Development of Antibodies against Interferon-β Therapy in Multiple Sclerosis

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The formation of antibodies to interferon-beta (IFN-β), a protein-based disease-modifying agent for multiple sclerosis (MS), is a problem in clinical practice. These antibodies may neutralize the biological effects of the protein drug, potentially decreasing its therapeutic effects. By high-resolution HLA class I and II typing we identified two HLA class II alleles associated with the development of antibodies to IFN-β. In two independent continuous and binary-trait association studies, HLA-DRB1*0401 and HLA-DRB1*0408 (odds ratio: 5.15)—but not other HLA alleles—were strongly associated with the development of binding and neutralizing antibodies to IFN-β. The associated HLA-DRB1*04 alleles differ from nonassociated HLA-DRB1*04 alleles by a glycine-to-valine substitution in position 86 of the epitope-binding alpha-helix of the HLA class II molecule. The peptide-binding motif of HLA-DRB1*0401 and *0408 might promote binding and presentation of an immunogenic peptide, which may eventually break T cell tolerance and facilitate antibody development to IFN-β. In summary, we identified genetic factors determining the immunogenicity of IFN-β, a protein-based disease-modifying agent for the treatment of MS.

Introduction

Multiple sclerosis (MS [MIM 126200]) is a chronic inflammatory disease of the central nervous system. Whereas the etiology of MS is likely to be heterogeneous, the influence of genetic factors in the development of MS has been confirmed by association and linkage studies.[1–4] The crucial role of HLA alleles in the susceptibility to MS is well established. Whereas HLA-DRB1*15 alleles are strongly associated with MS in Europeans, DRB1*0301 and DRB1*0401 are overrepresented in MS patients from southern Europe.[5,6] Overrepresentation of DRB1*04 alleles was reported for patients from Turkey and the Canary Islands. Although the genetic association of susceptibility to MS with loci outside the HLA complex has been confirmed in some cases, the search for susceptibility genes continues.[4]

Interferon-β is the most widely used immunomodulatory drug for MS therapy. It is approved for treatment of clinically isolated syndrome and relapsing-remitting MS. Furthermore, it has antiviral activity and remains an option in the therapy of diseases such as Hepatitis-C (HCV [MIM 609532]) or the severe acute respiratory syndrome (SARS).[7–9] Like other protein-based disease-modifying agents, IFN-β exhibits immunogenicity.[10] Even though the technical improvement of purification processes and the introduction of recombinant preparations have helped to reduce the immunogenicity of IFN-β, up to 50% of patients may develop antibodies to IFN-β (binding antibodies [BABs]), of which a significant proportion neutralize the activity of IFN-β (neutralizing antibodies [NABs]) in vitro

and vivo.[11] Currently, three distinct recombinant preparations of IFN-β are in use for the treatment of MS. Interferon β-1b (165 residues) is modified from naturally occurring IFN-β by deletion of the leading methionine and a single amino acid substitution (C17S).[12] It is recombinantly expressed in Escherichia coli (IFN-β-1b; Betaferon or Betaseron). IFN-β-1a, recombinantly expressed in Chinese hamster ovary cells, is identical to natural human interferon beta (166 residues) (IFN-β-1a; Avonex, Rebif). Although preparation and administration influence the immunogenic properties and the rate of antibody development, little is known about host factors that determine immune responses to IFN-β.[13]

Antibody production and generation of B cell responses to proteins depend on the help of antigen-specific CD4^{+} T cells.[14] The development of antigen-specific CD4^{+} T cell responses itself is strongly influenced by the individual repertoire of HLA class II molecules, which present peptide antigens for recognition to the T cell receptor.[15,16] Given the central role of CD4^{+} T cells for the development of antigen-specific B cell responses, we performed high-resolution typing for HLA-A, -B, -C, -DRB1, and -DQB1 alleles in MS patients receiving long-term IFN-β therapy.

Material and Methods

Patients

MS patients on IFN-β therapy, predominantly of northern European heritage, were recruited in Germany by primary-care physicians and neurologists. The initial study group included 268

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Table 1. Clinical Characteristics of the Initial Study and Validation Groups

Parameter	Initial Study Group (n = 268)				Validation Group (n = 242)			
	Anti-IFN-β-Positive Patients			Anti-IFN-β-Negative Patients (n = 132)	Anti-IFN-β-Positive Patients			Anti-IFN-β-Negative Patients (n = 132)
	All AB$^+$ (n = 136)	NAB$^+$ (n = 85)	BAB$^+$ (n = 51)		All AB$^+$ (n = 77)	NAB$^+$ (n = 59)	BAB$^+$ (n = 18)	
Age in years [mean (range)]	40 (17–69)	40 (19–64)	41 (17–69)	39 (16–66)	39 (21–75)	39 (21–60)	39 (21–75)	39 (16–67)
Gender (male/female)	48/88	27/58	21/30	45/87	23/54	19/40	4/14	40/124
Therapy duration in months [mean (range)]	55 (16–147)	53 (16–147)	57 (17–112)	59 (12–156)	47 (7–127)	47 (7–111)	46 (20–127)	55 (7–146)
IFN-β1a (i.m., Avonex)-treated (n)	6	5	1	28	4	2	2	64
IFN-β1b (s.c., Betaferon)-treated (n)	70	37	33	48	36	24	12	50
IFN-β1a (s.c., Rebif 22 μg)-treated (n)	10	6	4	16	9	9	0	15
IFN-β1a (s.c., Rebif 44 μg)-treated (n)	50	37	13	40	28	24	4	36
cELISA, antibody reactivity (%) [median (range)]	74 (26–119)	91 (35–119)	48 (26–97)	3 (−9–23.5)	83 (35–140)	85 (57–140)	76 (35–96)	2 (−17–24)
In vivo MXA assays, induction (%) [median (range)]	23 (−64–135)	−4 (−64–50)	89 (41–135)	92.6 (−12–93)	23 (−45–128)	17 (−45–57)	79 (53–128)	95 (70–202)

Both groups comprised a total of 510 IFN-β-treated subjects. Mean treatment duration exceeds 45 months in both cohorts. Ranges are given in brackets. Whereas the mean age of both groups was between 39 and 41 yr, the sex ratio differed among both groups. Females were slightly overrepresented in the validation group. Note that the number of BABs-positive subjects is higher in the IFN-β1b (Betaferon)-treated group.

individuals with known anti-IFN-β1 status. The ratio of males to females in the first cohort was 0.53. A total of 34 patients received IFN-β1a intramuscular injection (i.m.) once weekly (Avonex), 118 patients IFN-β1b subcutaneous injection (s.c.) every second day (Betaferon), 26 patients 22 μg and 90 patients 44 μg IFN-β1a s.c. three times a week (Rebif). The validation group compassed 242 patients with a male-female ratio of 0.36. Avonex treatment was administered to 68 patients, and Rebif was given to 88 individuals and 86 patients received Betaferon. As expected, IFN-β1a i.m. (Avonex)-treated patients were underrepresented in the study as a result of the lower frequency of antibody development in this group compared to patients treated with the formulations for s.c. application. Mean age was 39.8 yr in the first and 39.0 yr in the second group (Table 1). Average treatment duration was 57 months in the first and 52 months in the second group. The duration of the drug exposure did not differ significantly among treatment groups. All patients gave their written informed consent. The study was approved by the ethics committees of the Universities of Düsseldorf and Munich.

HLA Genotyping

For the first study cohort, HLA genotyping for *HLA-A*, *-B*, *-C*, *DRB1*, and *DQB1* was performed by hybridization of sequence-specific oligonucleotide probes immobilized on microspheres with amplified genomic DNA samples followed by flow fluorescence-intensity analysis according to the instructions of the manufacturer (One Lambda, Canoga Park, CA, USA). In the validation study cohort, the HLA analysis was restricted to *HLA-DRB1*04* only. The presence or absence of *HLA-DRB1*04* was determined by sequence-specific primer polymerase chain reaction (PCR-SSP) using a nested approach as previously described.[17] In both study cohorts, the identification of *HLA-DRB1*04* alleles was accomplished

by group-specific sequencing of exon 2 of *HLA-DRB1*04*-positive samples according to the manufacturer's instruction (Protrans, Ketschau, Germany).

Assays for Binding and Neutralizing Antibodies

To detect antibodies against IFN-β, we performed a standardized capture ELISA as described.[18] Defined positive and negative serum samples were included in each assay to obtain a standard curve for comparison of different assays. The standard curve ranged from 100% (highest positive control) to 0% (no antibodies). Serum positivity of all tested samples was normalized on the basis of these standard curves. Reactivity above 25% of the highest positive control was considered to be antibody positive (the cut-off value corresponded to the median of 50 control donors + five standard deviations). The biological in vivo activity of IFN-β was measured by *MX1* (MxA) gene expression with TaqMan real-time PCR.[19] Twelve hours after IFN-β injection, blood samples were drawn from subjects and mRNA was isolated according to the manufacturer's instructions. TaqMan reverse-transcription reagents (Applied Biosystems, Foster City, USA) were used to convert mRNA to cDNA. cDNA levels were detected with ABI's GeneAmp via the protocols described. The housekeeping gene GAPDH was used as internal control. Normalization was done according to the ΔΔCt-method. In each assay, the same positive and negative samples were included to ensure the quality of the assay. Antibodies were considered biologically active when the *MX1* (MxA) induction was decreased by more than 50% compared to newly treated antibody-negative control donors.

In 203 patients from the first patient group, an *MX1 RNA NAB* assay *was* also performed to determine the in vitro neutralizing antibody activity. A human lung carcinoma cell line (A549, American Type Culture Collection, #CLL-185) was diluted in culture

medium (2% BCS, 2 mM L-glutamine, 50 U/ml penicillin G, and 0.05 mg/ml streptomycin sulfate in alpha-MEM) at a concentration of 10^5 cells/ml, and each well of a microtiter plate was incubated overnight with 50 μl of this solution. Screening samples are diluted 1:10 and incubated with Betaferon 25 IU/ml for 45 min at 37°C and 5% CO2 on a microtiter plate. These samples are then transferred to the plate containing the A549 cells and incubated for 6.5 hr. For the standard curve, Betaferon with concentrations from 0 to10 IU was used. After that, the supernatant was decanted and cells were lysed with a lysis buffer.

A ΔCt value of a sample of less than 50% of the highest concentration on the standard curve was considered NAB negative. Otherwise, the sample was titrated. Titration samples were diluted serially beginning at 1:20 up to 1:10,240, and each dilution was incubated with Betaferon 25 IU/ml for 45 min at 37°C and 5% CO2 with an extra microtiter plate. These samples were then transferred to the plate containing the A549 cells and incubated for 6.5 hr. The supertnatant was decanted and cells were lysed with a lysis buffer followed by RNA extraction and transcription as described below. *MX1* RNA was isolated semiautomatically with 6100 ABI PRISM Nucleic Prep Station. Lysates were filtered through a membrane to capture RNA. After several washing steps with washing buffers (by Applied Biosystems), RNA was eluted by addition of a purification elution solution into an extraction plate and stored at −80°C. After Master Mix was prepared (high-capacity cDNA Kit by Applied Biosystems) and applied to a new microtiter plate, the samples from the above RNA plate were thawed and transferred. RNA was transcribed into cDNA according to the manufacturer's instructions. Endogenous control (housekeeping gene) and target-detecting primers were mixed with the appropriate Master Mix (Applied Biosystems), and c-DNA was added. A predefined program (Applied Biosystems) for *MX1* and house keeping gene (eukaryotic 18 s rRNA) detection was applied. A threshold is set in the exponential phase of rtPCR and the difference between the *MX1* threshold cycles and the housekeeping gene cycles was determined (= ΔCt).

Computational Biology and Statistics

In order to attenuate the problem of deliberately setting up thresholds for ELISA and *MX1* values to declare cases and controls, a binary-trait case-control association study was supported by continuous trait analysis for single HLA alleles and haplotypes. This bilateral approach was used for the initial study group as well as for the validation group. For identification of genetic variation of anti-IFN-β formation, the continuous normalized antibody reactivities were directly compared among carriers of different alleles. In the binary-trait case-control study, patients were considered antibody positive (cases) if the anti-IFN-β levels exceeded the 25% cutoff value. Otherwise, patients were considered antibody negative (controls). Additionally, antibody-positive patients were considered NABs positive if their *MX1* mRNAs were reduced below 50% of *MX1* levels of newly treated patients. The remaining antibody-positive patients who had not significantly reduced *MX1* mRNA levels were considered BABs positive. For the testing of differences of location parameters for *MX1* gene expression and anti-IFN-β levels, the two-sided nonparametric Wilcoxon test was applied. p values were adjusted for multiple testing with the false-discovery rate (FDR). For identification of haplotype-specific associations with continuous (*MX1* and anti-IFN-β levels) and binomial trait types (NABS, BABs, and control subgroups), the genetic analysis package (gap) of the R Project for statistical computing was applied with score tests for association as described

by [20,21]. Haplotype-specific scores have been calculated with a score test for ambiguous haplotypes. The score statistics are given by

$$U_\beta = \sum_{i=1}^{N} \frac{(y_i - \bar{y}_i)}{a(\phi)} E_p(X_{gi})$$

where y_i denotes the measured trait, $\bar{y}_i$ a mean trait value, and $a(\phi)$ a measure of variation. $E_p(X_{gi})$ denotes expectation value over the posterior distribution of the null hypothesis. The score statistics provide a measure for the covariation of a haplotype and the examined trait.[21] However, this analysis has explorative character, because the results are based on the maximization of probabilities rather than a known linkage phase.

Multiple sequence alignments were generated with the CLUSTALW application. The sequences of HLA alleles were obtained from the HLA repository at the European Bioinformatics Institute (EBI). Linkage disequilibrium (LD) tests for HLA alleles as well as multilocus analysis for LD were done with the PyPop package.

Results

HLA Class II but Not HLA Class I Alleles Are Associated with Antibody Occurrence

Two hundred and sixty-eight MS patients on long-term IFN-β therapy were included in the first group of the study. One hundred and thirty-six patients of the study group had developed antibodies to IFN-β (determined by cELISA), of whom 85 developed neutralizing antibodies as determined by the in vivo induction of the IFN-β response gene *MX1*. One hundred and thirty-two patients were antibody negative (Table 1). In the second group, 76 patients were antibody positive, including 59 with neutralizing in vivo activity. The remaining 164 individuals were antibody negative. For analysis of possible confounding effects, treatment-independent allele distribution was tested. The null hypothesis of independency holds in the study (chi-square = 55.34, p value = 0.65) as well as in the validation group (chi-square = 8.333, p value = 0.97).

All patients from the initial study group were typed for HLA class I and II alleles. Although we expected an association of HLA class II genes with antibody development, we also typed class I genes as internal negative control. As expected, only small differences between antibody-negative and -positive patients were found for HLA class I alleles. Median anti-IFN-β levels were lower in *HLA-C*16* carriers (p value = 0.007, FDR = 0.095) and higher in *HLA-C*2* and *HLA-B*57* (p value = 0.03, FDR ≤ 0.1) carriers, although the associations did not reach significance after adjustment for multiple testing.

By contrast, significant differences were observed for the HLA class II alleles. The largest difference of median anti-IFN-β levels was observed between *DRB1*04*-positive (n = 58) and *DRB1*04*-negative subjects (n = 210) (p = 0.006). *DRB1*04*-positive patients showed significantly higher anti-IFN-β levels with a median normalized antibody titer of 55.1% compared to *DRB1*04*-negative patients (median = 17.1%) (Table 2). Significantly reduced median anti-IFN-β reactivities were also found for *DRB1*1101*

Table 2. Anti-IFN-β Levels and Low-Resolution HLA Class II Typing in the Study Group

Specificity	Allele Carriers[a] n	Anti-IFN Reactivity (%)[b] Median	Range	Statistics p[c]	FDR[d]
DR 1	31	14.6	−0.7–100.5	0.2	0.43
DR 3	48	17.9	−3.2–110.8	0.34	0.55
DR 4	**58**	**55.9**	**−3.2–119.3**	**0.004**	**0.025**
DR 7	50	18.2	−9.2–119.3	0.41	0.59
DR 8	16	37	0.2–103.5	0.87	0.87
DR 10	5	6.7	1.8–43.4	0.14	0.43
DR 11	**40**	**6.3**	**−4.5–112.1**	**0.003**	**0.02**
DR 12	7	46.6	−4.7–78.0	0.69	0.75
DR 13	53	17.5	−9.22–110.8	0.2	0.43
DR 14	4	41.8	9.92–91.2	0.55	0.65
DR 15	155	28.3	−4.6–112.1	0.26	0.48
DR 16	**21**	**90.8**	**0.0–103.0**	**0.006**	**0.03**
DQ 2	90	17.7	−9.2–110.8	0.1	0.5
DQ 3	121	33.7	−4.7–119.3	0.99	0.99
DQ 4	15	54.4	0.3–103.5	0.46	0.74
DQ 5	60	18.3	−9.2–102.9	0.59	0.74
DQ 6	180	28.3	−4.6–112.1	0.28	0.7

The anti-IFN-β levels are compared among different HLA class II alleles. Median anti-IFN-β levels are significantly elevated in patients carrying the DR4 and DR16 alleles (in bold). Subjects carrying the DR11 serotype show a significant decrease of anti-IFN-β, indicating a protective effect of DR11 alleles (in bold). Significant results for HLA-DQ serotypes were not observed.

[a] For all alleles listed, note that heterozygous allele carriers occur twice, and homozygotic allele carriers occur only once. Only alleles that occurred more than twice are shown.

[b] Anti-IFN reactivity was determined as described in the Material and Methods. Values above 25% identified patients with anti-IFN antibodies.

[c] p value was determined by comparison of the anti-IFN level of allele carriers to that of all nonallele carriers.

[d] p values have been adjusted to multiple testing by using the false-discovery rate method (FDR). Adjusted p values below 0.05 were considered to be statistically significant (in bold).

(p value = 0.013) and *DRB1*1104* (p value = 0.02). However, null hypothesis was kept after the FDR procedure. Likewise, elevated anti-IFN-β reactivities in *DRB1*1601*-positive patients (p value = 0.02) were not significant after correction.

HLA-DRB1*04 Alleles Confer a Higher Risk of Developing Antibodies during IFN-β Therapy

A majority of *DRB1*04*-positive patients included in the initial study group carried the *DRB1*0401* allele (n = 42), whereas only 12 subjects were *DRB1*0404*-positive. Only four patients carried *DRB1*0408* (Table 3). Differences of location parameters for *DRB1*0401* (FDR = 0.012) and *DRB1*0408* (FDR = 0.048) were significant after correction for multiple testing. On the other hand, average cELISA levels for *DRB1*0404* were lower compared to *DRB1*04*-negative patients. After FDR correction, null hypothesis was kept for *DRB1*0404* despite a significant single test (p value = 0.031, FDR = 0.16).

To further investigate the role of DR4 alleles for antibody development, we determined the activity of IFN-β antibodies by another assay, which was used in a number of clinical studies. The in vitro *MX1* test was performed on

203 randomly selected samples. As expected, a significant increase (p value = 0.003) of neutralizing in vitro activity was found in sera of *HLA-DRB1*0401*- and *0408*-positive subjects (median activity = 8462 NU) compared to sera from *HLA-DRB1*0401*- and *0408*-negative patients (median activity = 0) (data not shown).

For validation of our initial findings, we used a second study cohort and analyzed the most significant results for *DRB1*04*. HLA genotyping of the validation group supported the previous findings. Fifty-nine of 242 individuals were *DRB1*04* positive. *DRB1*0401* carriers showed significantly higher levels of anti-IFN-β antibodies. Median cELISA positivity was 71.8% (p value = 0.005, FDR = 0.03) (Table 3). Five subjects carried the *DRB1*0408* allele, with a median level of 91.6%. Whereas the elevation of ELISA reactivity was significant in a single test, null hypothesis was kept after correction for multiple testing (p value = 0.04, FDR = 0.15).

In a combined analysis of anti-IFN-β levels for DR4-positive and DR4-negative carriers of validation and study groups, a highly significant elevation of anti-IFN-β levels was found in carriers of *DR4* alleles (median reactivity = 55.1%, p value ≤ 0.01) (Figure 1). Fifty-four of 74 *HLA-DRB1*0401* carriers showed antibodies during IFN-β treatment. In *DRB1*0401*- and *0408*-negative subjects, 153 of 429 showed seroconversion, resulting in an odds ratio of 4.9 for *HLA-DRB1*0401*-positive carriers. The odds ratio for *DRB1*0408* was 14.4, with four of five allele carriers being antibody positive. A highly significant increase of ELISA reactivities was observed in a comparison of all HLA-*DRB1*0401*- and *0408*-positive carriers versus HLA-*DRB1*0401*- and *0408*-negative carriers (Figure 2) (p value ≤ 0.01). Furthermore, the difference of anti-IFN-β levels among *DRB1*0401* (median reactivity = 70.6%) and *DRB1*0408* (median reactivity = 96.4%) is significant (p value = 0.006). Because of the small number of homozygous *DRB1*04* carriers, a comparison with *DRB1*04*-negative or heterozygous carriers was not possible.

The haplotype analysis was performed with score statistics for ambiguous haplotypes. DRB*11 alleles might have a protective influence: DRB1-DQB1 haplotype analysis shows a significant reduction of anti-IFN-β levels in *DRB1*1101-DQB1*0301* and *DRB*1104-DQB1*0301* carriers. Elevated *MX1* mRNA levels in carriers of these haplotypes support this observation. A significant increase of anti-IFN-β reactivity could be observed for *DRB1*0401-DQB1*0301* and *DRB1*0401-DQB1*0302* haplotypes. The reduction of *MX1* mRNA levels, however, was not significant (p value ≥ 0.5). Reduced *MX1* levels as well as increased antibody reactivities were significantly changed in patients carrying the *DRB1*0408-DQB1*0301* haplotype. Susceptibility to seroconversion was increased for *DRB1*1601-DQB1*0502* carriers. The *DRB1*1601-DQB1*0502*-positive carriers also showed significantly decreased *MX1* levels (Table 4).

As expected pairwise linkage disequilibrium was significant for all observed major histocompatibility complex (MHC) loci (p value ≤ 0.001). Strongest disequilibria

Table 3. Anti-IFN-β Levels for Alleles of the *HLA-DRB1* Locus in the Study and Validation Groups

DRB1	Allele Carriers[a,b] n	Anti-IFN Reactivity (%)[c] Median	Range	Statistics p[d]	FDR[e]
Inital Study Group					
0101	27	10.8	−0.83–100.5	0.12	0.45
0301	48	17.8	−3.16–110.8	0.34	0.66
0401	**42**	**66.6**	**−3.18–119.3**	**4e-04**	**0.012**
0404	12	6.6	−1.75–96.54	0.031	0.16
0408	**4**	**98.5**	**90.5–109.8**	**0.003**	**0.048**
0701	50	18.2	−9.2–119.3	0.41	0.66
0801	15	39.4	0.2–109.5	0.74	0.8
1001	5	6.7	1.79–43.4	0.14	0.47
1101	32	27.0	−4.6–112.1	0.013	0.13
1104	8	3.1	−0.6–48.62	0.02	0.14
1201	7	46.6	−4.8–78.0	0.69	0.8
1301	25	38.1	−2.1–102.9	0.74	0.8
1302	18	15.8	−9.2–110.8	0.35	0.66
1303	12	11.9	−1.61–81.7	0.24	0.6
1501	153	30.5	−4.6–112.1	0.43	0.66
1502	3	87.3	3.4–103.2	0.31	0.66
1601	17	90.8	0.1–102.9	0.02	0.13
1602	3	77.6	3.9–95.29	0.44	0.66
Validation Group					
DR4[−]	183	5.5	−17.3–140.3	0.12	0.21
DR4[+]	**59**	**10.1**	**−8.3–123.1**	0.12	0.21
0401	**31**	**71.8**	**−5.4–120.4**	**0.005**	**0.03**
0402	6	0.3	−7.0–23.17	0.01	0.21
0403	6	3.4	−8.3–89.6	0.37	0.43
0404	9	1.9	−5.9–84.1	0.22	0.31
0407	2	47.8	2.8–92.8	0.51	0.51
0408	5	91.6	−3.7–123.1	0.04	0.15

A significant elevation of IFN-β reactivity is seen for *DRB1*0401* and *DRB1*0408* (in bold) after adjustment for multiple testing. Only alleles that occurred more than twice are shown. In contrast to the findings in the low-resolution analysis, a significant increase of anti-IFN-β levels in *DRB1*1601*-positive subjects vanishes after the FDR procedure. Likewise, the potential protective effect of *DRB1*0404* does not pass the significance criterion after adjustment for multiple testing. In the validation group, a significant elevation of IFN-β reactivity is seen in single tests for *DRB*0401* (in bold) as well as for *DRB1*0408*. Because of an insufficient number of cases, however, significance vanishes after correction for multiple tests for *DRB1*0408*. Because of the small number of cases, a potential protective effect of *DRB1*0402* did not pass the FDR significance criterion. The results in the validation group support our previous findings.

[a] For all alleles listed, note that heterozygous allele carriers occur twice, and homozygotic allele carriers occur only once. Only alleles that occurred more than twice are shown.

[b] Heterozygous allele carriers (*0401[+] and *0408[+]) occur twice.

[c] Anti-IFN reactivity was determined as described in the Material and Methods. Values above 25% identified patients with anti-IFN antibodies.

[d] p value was determined by comparison of the anti-IFN level of allele carriers to that of all nonallele carriers.

[e] p values have been adjusted to multiple testing by using the false-discovery rate method (FDR). Adjusted p values below 0.05 were considered to be statistically significant (in bold).

were found for *HLA-DRB1:DQB1* (D′ = 0.94), *HLA-B:C* (D′ = 0.92), and *HLA-B:DRB1* (D′ = 0.68). Haplotype frequencies for HLA class I alleles with significant increases of anti-IFN-β reactivities in single test (Table S1 available online) do not indicate a common transmission with *DRB1*0401* and *DRB1*0408* (data not shown).

*HLA-DRB1*04* Promotes the Development of Binding and Neutralizing Antibodies Independent of the IFN-β Formulation

Whereas *DRB1*0401* and *DRB1*0408* are significantly associated with the presence of antibodies, *HLA-DRB1*0401* and **0408* frequencies were not different between patients who developed NABs or BABs (p value > 0.05) (Table 5), sug-

gesting that the antibody development itself but not their neutralizing capacity is influenced by the HLA alleles. The rejection of the alternative hypothesis might, however, be caused by an insufficient power of the test.

The odds ratios in the study and validation group for the development of antibodies to IFN-β were 5.15 for *HLA-DRB1*0401*-positive patients and 9.6 for *HLA-DRB1*0408*-positive patients. Odds ratios were increased for all IFN-β preparations, although they did not reach significance in the IFN-βa i.m.-treated and IFN-βa 22 μg s.c.-treated patients as a result of the low numbers of patients in these treatment groups (odds ratios were 3.2 and 8, respectively). The strongest effect was observed in patients who received IFN-β1b s.c. treatment (Table 5).
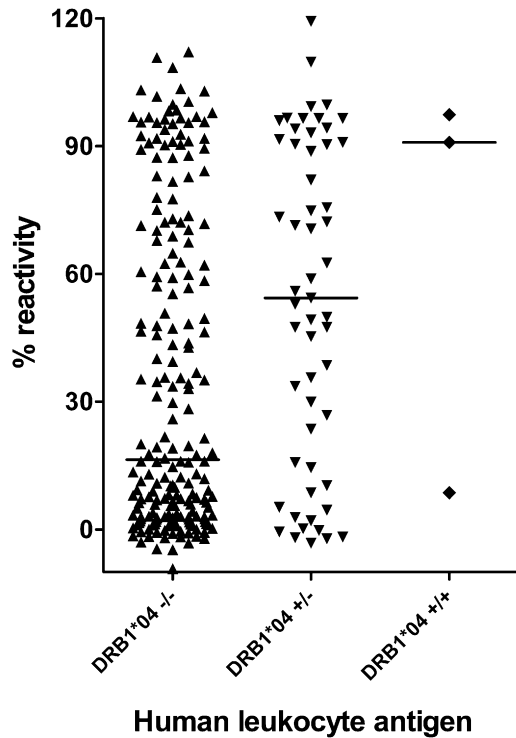
Figure 1. Comparison of Anti-IFN-β Reactivities for HLA-DR Serological Equivalents in the Study Group
Median ELISA optical density (OD) of heterozygous DR4 carriers was significantly elevated compared to DR4-negative individuals (p value ≤ 0.01).

HLA Alleles Associated with Antibody Development Share a Similar Peptide-Binding Motif

To further investigate the molecular basis for HLA class II-restricted antibody development, we compared the sequences of the antibody-associated *DRB1*0401* and *DRB1*0408* to the *DRB1*0402*, *DRB1*0403*, and *DRB1*0404* alleles that are not associated with antibody development. *DRB1*0401* and *DRB1*0408* differ by one amino acid (71 K→R). *DRB1*0401* and *DRB1*0408* differ from *DRB1*0404*, *DRB1*0402*, and *DRB1*0403* at position 86, which forms the first pocket of the peptide-binding groove located at the terminal end of the same alpha-helix (Figure 3). Both alleles associated with antibody development share a glycine at position 86, whereas the nonassociated alleles have a valine at this position (86 G→V).

Discussion

As of December, 2006, 65 new biopharmaceuticals had been approved in the U.S., European Union, and Japan since 1999. Like IFN-β, 47 of these new biopharmaceuticals are derived from recombinant DNA technology.[22] Applications range from the treatment of more prevalent rheumatic diseases (Ethanercept) to rare acquired or inherited disease with protein deficiencies (Galsulfase). It is predicted that many more therapeutic proteins will become available in the near future for an even wider range of indications.[10] Despite their therapeutic potential, immunogenicity is a common problem to most, if not all, protein-based biopharmaceuticals and an important aspect of their safety. Antibodies might not only lead to the loss of drug efficacy but may also induce severe allergic reactions or neutralize the activity of endogenously produced protein.[23] It has been argued that in the absence of resilient predictors of immunogenicity, patients might be put at a high risk.[24] Preparation, handling, packaging, dose, frequency, and route of administration and treatment duration seem to affect immunogenicity of biopharmaceuticals.[25–27] However, the role of genetic factors in
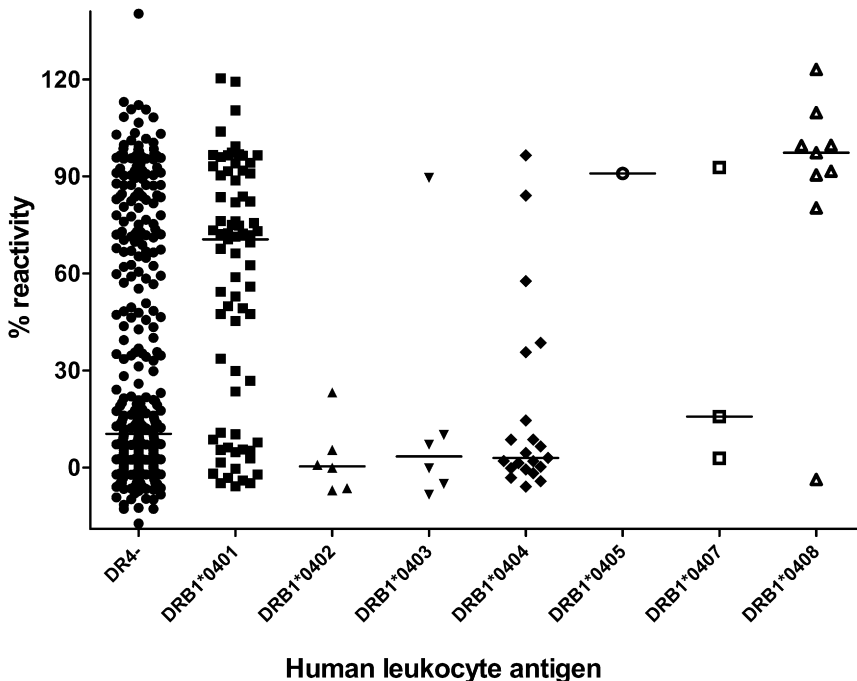

Figure 2. Comparison of Anti-IFN-β Reactivities for *HLA-DRB1* Alleles in the Study and Validation Groups
A significant increase of ELISA reactivities was measured for *HLA-DRB1*0401* and *HLA-DRB1*0408* alleles (p value ≤ 0.01). *DRB1*0402*, *DRB1*0403*, and *DRB1*0404* seem to have a protective effect.

Table 4. Haplotype Analysis for the HLA Class II Locus

Haplotype			MxA mRNA Levels (% Positivity)		Anti-IFN Reactivity (%)	
DRB1	DQB1	Freq	Score	p	Score	p
0101	0501	0.05	1.3	0.2	−1.93	0.05
0301	0201	0.10	0.2	0.8	−0.68	0.5
0401	**0301**	**0.02**	**−0.7**	**0.5**	**2.05**	**0.04**
0401	**0302**	**0.06**	**−1.4**	**0.2**	**3.35**	**8e-04**
0404	0302	0.02	0.9	0.4	−2.13	0.03
0408	**0301**	**0.01**	**−2.6**	**0.01**	**2.76**	**0.006**
0701	0201	0.09	0.2	0.8	−0.7	0.5
0701	0303	0.01	−0.2	0.8	0.63	0.5
0801	0402	0.03	0.8	0.4	0.25	0.8
1001	0501	0.01	1.1	0.3	−1.61	0.1
1101	**0301**	**0.06**	**2.8**	**0.004**	**−2.16**	**0.03**
1104	**0301**	**0.02**	**2.1**	**0.04**	**−2.23**	**0.03**
1201	0301	0.01	0.1	0.9	0.49	0.6
1301	0603	0.04	−0.7	0.5	0.15	0.9
1302	0604	0.02	−0.6	0.5	−0.44	0.7
1302	0609	0.01	−2.1	0.03	1.68	0.09
1303	0301	0.02	1.4	0.2	−1.58	0.1
1501	0602	0.30	−0.5	0.7	0.54	0.6
1501	0603	0.02	−0.5	0.6	0.13	0.9
1501	06XX	0.01	0.4	0.7	−1.14	0.3
1502	0601	0.01	−0.9	0.4	1.16	0.2
1601	**0502**	**0.03**	**−2.7**	**0.006**	**2.77**	**0.006**
1602	0502	0.01	−1.1	0.3	0.9	0.4

A significant increase of anti-IFN-β activities can be observed for haplotypes containing *HLA-DRB1*0401, DRB1*0408* and *DRB1*1601* alleles, *HLA-DRB1*11* and DRB1*0404 alleles have a protective effect on seroconversion (in bold). Additionally, for *DRB1*1601* and *DRB1*0408* a significant reduction of *MX1* levels is seen. Interestingly, even though not significant in single allele tests, the haplotype *DRB1*1601-DQB1*0502* shows a significant reduction of *MX1* levels and elevation of anti-IFN-β levels. Scores reflect the strength and the direction of the effects. The % reactivity of each sample is related to the highest measured OD of positive controls (MS patients with high titers of anti-IFN-β).

immunogenicity to protein-based therapeutics has not been addressed. In the present study we show that—besides the influence of different preparations on immunogenicity—genetic factors play an important role in the development of antibodies against protein therapeutics. Patients who carry the *HLA-DRB1*0401* or *HLA-DRB1*0408* allele show a 5- and 14-fold increased risk to develop antibodies to IFN-β, respectively. This results in an average relative risk of 2.07 for *DRB*0401*- and

Table 5. Odds Ratios for *HLADR*0401*- and *HLADR*0408*-Positive Allele Carriers

		Initial and Validation Study Group: Allele Carriers		
Treatment	AB Status	0408+ and 0401+	0408− and 0401−	Odds Ratio (p Value)
All Products				
	NABs	37	107	
	BABs	23	46	0.7 (> 0.05)
	ABs	60	153	
	no ABs	21	276	5.2 (< 0.0001)
IFN-β1a (i.m., Avonex)				
	ABs	3	7	
	no ABs	11	81	3.2 (> 0.1)
IFN-β1b (s.c., Betaferon)				
	ABs	36	70	
	no ABs	5	93	9.6 (< 0.0001)
IFN-β1a (s.c., Rebif 22 μg)				
	ABs	4	15	
	no ABs	1	30	8 (0.06)
IFN-β1a (s.c., Rebif 44 μg)				
	ABs	17	61	
	no ABs	4	72	5 (< 0.005)

The difference of NABs and BABs cases among *DRB1*0401*- and *DRB1*0408*-positive patients was not significant in chi-square tests for the study (p = 0.19) as well as the validation group (p = 0.50). Odds ratios for the development of antibodies are increased irrespective of the type of the IFN-β preparation. This observation is true for the study as well as for the validation group. Overall, the relative risk (RR) for *DRB1*0401*- and *DRB1*0408*-positive carriers was 1.85 in the study and 2.43 in the validation group. Because of missing observations (no antibody-negative patients with *HLA-DRB1*0401* or *HLA-DRB1*0408*) in the validation group, the exact odds ratio could not be calculated for IFN-β1a (s.c., Rebif 22 μg).

*DRB*0408*-positive patients. Although the *HLA-DRB1*0401* or *HLA-DRB1*0408* alleles increase the risk of developing antibodies in response to all IFN-β formulations, it remains to be determined, in further studies and with a different study design, whether formulations with higher immunogenicity are more affected by the HLA haplotype than those with low immunogenicity.

Figure 3. Multiple Sequence Alignment of *DR4* Alleles

*HLA-DRB1*0401* and *DRB1*0408* only differ in position 71 (site A). In contrast to protecting DR4 alleles, both sequences encode for a glycine in position 86 (site B). The residue is part of the C-terminal end of the epitope-binding alpha helix of the MHCII complex. The substitution of a large with a small hydrophobic amino acid at this position potentially increases the immunogenicity of IFN-β.

These results are in line with the central role of T cells in eliciting B cell responses to protein targets: It seems that the peptide-binding motif of both alleles allows processing and binding of an IFN-β peptide that breaks tolerance by promoting a strong T helper cell response to the IFN-β protein. This breakdown of tolerance promotes a B cell response that eventually leads to the development of an antibody production against the IFN-β protein. It is striking that this effect critically depends on a single amino acid in the HLA-DRB1*04 allele sequence. The 86 G→V substitution in HLA-DRB1*04 alleles is likely to interfere with elevated immunogenicity either by altering the binding affinity of the IFN-β peptide because of secondary structure changes of the epitope-binding alpha helix or by affecting the presentation of the IFN-β peptide itself. It seems that these two alleles influence the general likelihood of developing an antibody response independent of the IFN-β preparation. Although antibody development was strongly affected by the alleles, the neutralizing activity of the antibodies was not significantly influenced by these HLA alleles. This suggests that both HLA-DRB1*04 alleles increase the likelihood of breaking tolerance to the protein by antibody production. Other factors, however, might determine whether the antibodies will neutralize IFN-β. One of those factors could be the intensity of an immune response to IFN-β. However, the rejection of the alternative hypothesis that the development of neutralizing antibodies is determined by HLA-DRB1*04 alleles might be due to insufficient test power and remains to be clarified in further studies.

Interestingly, HLA-DRB1*0401 has been associated with the development of autoantibodies against insulin. Congia et al. identified an immunodominant epitope in DRB1*0401 transgenic mice that would have been proteolytically destroyed under normal conditions. Additionally, this epitope was processed and presented by human DRB1*0401-positive Epstein-Barr virus-transformed B cells.[28] First reports on the influence of HLA-DR4 serotypes in the development of porcine insulin antibodies date back to the early 1980s.[29] The smaller glycine might endorse the binding of epitopes that contain larger amino acids in the vicinity of the substitution site or promote binding by a slight modification of the secondary structure. Although HLA-DRB1*0101 is similar to HLA-DRB1*0401 (both carry a glycine at position 86) and has analogous disease-modifying properties in MS, only HLA-DRB1*0401 influences antibody development to IFN-β.[30,31] Although not significant in single-allele tests, the explorative haplotype analysis based on a score test for unkown linkage phase reveals a significant reduction of MX1 production in DRB1*0408-DQB1*0301 carriers. Additionally, haplotype analysis showed a significant increase of anti-IFN-β reactivity and reduced MX1 mRNA expression in DRB1*1601-DQB1*0502 carriers, pointing to alleles other than DRB1*0401 and DRB1*0408 that might be associated with the development of binding and neutralizing antibodies. This is especially true for HLA-DRB1*16 alleles that also potentially increase the risk to develop antibodies against IFN-β. On the other hand, DRB1*1101 alleles seem to have a protective effect with respect to antibody production. It has been recently shown that trans interactions of HLA-DRB1 alleles play a significant role in the susceptibility to MS.[32,33] Such trans interactions might also play a role in the immunogenicity of biotherapeutics. Hence, this study suggests that further genetic studies should be undertaken to clarify the influence of hereditary factors in immunogenicity of biotherapeutics and improve treatment decisions.

Supplemental Data

Supplemental Data include one table and can be found with this article online at http://www.ajhg.org/.

Web Resources

The URLs for data presented herein are as follows:

IMGT/HLA database, http://www.ebi.ac.uk/imgt/hla
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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