Major histocompatibility locus genetic markers of beryllium sensitization and disease

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ABSTRACT: Hypersensitivity to beryllium (Be) is found in 1–16% of exposed workers undergoing immunological screening for beryllium disease using the beryllium lymphocyte proliferation test (BeLPT). However, only ~50% of BeLPT-positive workers present with lung granulomas (*i.e.* berylliosis). As berylliosis is associated with the human leukocyte antigen (HLA)-DP supratypic marker DPGlu69, the authors asked whether this marker is differentially associated with disease presentation.

A population of 639 workers from a beryllium factory undergoing BeLPT screening was evaluated in a nested case-control study for the prevalence of HLA-DPGlu69, the HLA-DPB1, HLA-DQ and HLA-DR alleles and of the biallelic tumour necrosis factor (TNF)-α polymorphism TNF-α-308 in 23 individuals presenting as "sensitized" (*i.e.* BeLPT-positive without lung granulomas) and in 22 presenting as "diseased" (*i.e.* BeLPT-positive with granulomas in the lung biopsy).

The HLA-DPGlu69 marker was associated with "disease" (odds ratio (OR) 3.7, p=0.016, 95% confidence interval (CI) 1.4–10.0), whilst the high TNF-α production-related TNF-α-308*2 marker was associated with both a positive BeLPT (OR 7.8, corrected p<0.0001, 95% CI 3.2–19.1) with no difference between "sensitization" and "disease". Furthermore, the HLA-DRArg74 marker was associated with "sensitization" without disease (OR 3.96, p=0.005, 95% CI 1.5–10.1).

The data indicate that tumour necrosis factor-α, human leukocyte antigen-DR and human leukocyte antigen-DP markers play different roles in beryllium sensitization and granuloma formation in beryllium-exposed workers.
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Hypersensitivity to beryllium (Be) in workers exposed to Be dusts and fumes is the cause of a spectrum of reactions ranging from acute pneumonitis to berylliosis, a chronic granulomatous disease primarily affecting the lung [1].

Following the observation that blood T-cells of berylliosis patients proliferate in response to Be *in vitro*, a beryllium-stimulated blood lymphocyte proliferation test (BeLPT) has been developed to identify Be hypersensitive workers [2]. Screening programmes including the BeLPT and a lung transbronchial biopsy (TBB) showing noncaseating granulomas to diagnose berylliosis, have been effective in the earlier identification of affected individuals, as "screening" identified berylliosis cases showed milder disease than those identified upon clinical complaints or the finding of an abnormal chest radiograph [3]. Among the subjects showing a positive blood BeLPT, only 40–60% presented with a TBB positive for granulomas (*i.e.* berylliosis). A yet undetermined fraction of those with a negative TBB may eventually develop lung granulomas [4].

Berylliosis risk has been consistently associated with the expression of the supratypic human leukocyte antigen (HLA)-DPB1Glu69 (DPGlu69) marker, a marker that has been found to be expressed in 84–97% of disease cases in three separate studies [5–7], and that has been shown to function as the restriction element for Be presentation to Be-specific T-cell clones from HLA-DPGlu69-positive subjects

with berylliosis (*i.e.* as the immune response gene of the Be-specific T-cell reaction of berylliosis) [8].

Disease presentation has been associated with major histocompatibility complex (MHC) locus gene markers in sarcoidosis, a granulomatous disorder similar to berylliosis, where spontaneously resolving hilar adenopathy has been associated with HLA-DR3 and chronic fibrosing lung disease with HLA-DR5 [9]. In this context, it was hypothesized that the DPGlu69, as well as other MHC markers, could be associated with disease presentation in Be-sensitized workers. Hence, a case-control study was designed including all BeLPT-positive subjects who were identified in the course of the screening of a large beryllium manufacturing plant. This sensitized group was further subdivided into: 1) a blood BeLPT-positive, TBB-negative subject or "sensitized" subgroup and 2) a BeLPT-positive, TBB-positive subject or "disease case" subgroup.

Methods

Study population and case definition

A population of 639 individuals from a beryllium manufacturing plant in Midwestern USA was enrolled after informed consent into a genetic study for susceptibility to berylliosis, in conjunction with the enrollment into a beryllium disease surveillance programme carried out by the plant′s Medical Department, which included blood BeLPT carried out in two laboratories following published protocols [4]. This population had been described previously by KREISS *et al.* [10]. For the purpose of the present study, individuals with two abnormal BeLPTs in two separate blood samples were defined as BeLPT-positive or "sensitized" and were offered medical evaluation. The diagnosis of berylliosis was established upon a fibreoptic bronchoscopic TBB showing noncaseating granulomas. Individuals with a single abnormal BeLPT and "sensitized" individuals who declined medical evaluation were not included in the study. Twenty-three BeLPT-positive TBB-negative individuals were identified and defined as "sensitized"; they included 21 males and two females, mean±SD age 42±9 yrs, average years of employment 14±9, all Caucasians. Twenty-two BeLPT-positive TBB-positive individuals were identified and defined as "disease cases"; they included 21 males and one female, mean±SD age 41±8 yrs, average years of employment 15±6, 20 Caucasians and two African-Americans. Ninety-three Be-exposed BeLPT-negative individuals were randomly selected from the whole Be-LPT-negative workers on the basis of a nested case-control study design, and therefore evaluated as controls; they included 78 males and 15 females, mean±SD age 44±9 yrs, average years of employment 16±11, 88 Caucasians, two Hispanics, two African-Americans, one Asian. The sample of the control population was selected on a 1:2 ratio and on the basis of previously published HLA-DP gene association data, in order to assure a sufficient statistical power to the study. The "sensitized" individuals and

the "disease cases" were not statistically different from the control group for sex, age, time on the job and race.

Genetic studies

The study population was evaluated for the frequencies of two tumour necrosis factor (TNF)-α alleles (-308 biallelic polymorphism), TNF-α-308*1 and TNF-α-308*2 and HLA class II (HLA-DPB1, HLA-DQB1, HLA-DRB1 and HLA-DRB3/4/5) alleles.

Tumour necrosis factor-α gene polymorphism analysis

The analysis of the biallelic polymorphism (G to A) at position -308 of the promoter of the TNF-α gene was carried out by polymerase chain reaction (PCR) amplification of 500 ng of genomic deoxyribonucleic acid (DNA) using the primers TNF-α-308-A (CA AACACAGGCCTCAGGACT) and TNF-α-308-B (AGGGAGCGTCTGCTGGCTG) with the following PCR cycles (GeneAmp 9600, Perkin Elmer Corporation, Roche Molecular Systems, Branchburg, CT, USA): one denaturation cycle (95°C for 1 min with a pause at 80°C for the hot start technique), 30 amplification cycles (94°C for 30 s, 55°C for 30 s, 72°C for 30 s), one extension cycle (72°C for 7 min). The expected molecular weight of the amplified product was 545 base pairs (bp). Ten μL of the PCR product were then analysed on a 2% agarose gel stained with ethidium bromide. Twenty μL of the PCR product were probed using the oligonucleotide probes TNF-1 (Biotin-AGGGGCATGGGGACGGG) and TNF-2 (Biotin-AGGGGCATGAGGACGGG) in the nonradiometric hybridization DNA enzyme immunoassay (DEIA) method (Gen-ETI-K DEIA, Sorin Biomedica, Saluggia, Vercelli, Italy). The DEIA experimental conditions were as follows: PCR product: 20 μL PCR product per well; probe concentration: 5 ng probe per well; hybridization conditions: 1 h at 50°C. The hybridization results were obtained as optical density values on an enzyme-linked immunosorbent assay (ELISA) fast reader (Sorin Biomedica). In order to confirm the hybridization results, 20 DNA samples were sequenced for the TNF-α-308 genotyping. Briefly, 250 ng of genomic DNA were PCR amplified with the primers TNF308AM13 (TGTAAAACGACGGCCAGTGAAACAGACCAC AGACCTG) and TNF308BB (Biotin-CTTCTGTC TCGGTTTCTTC) using one denaturation cycle (95°C for 1 min), 35 amplification cycles (94°C for 1 min, 54°C for 20 s, 72°C for 20 s), one extension cycle (72°C for 7 min) to generate a 250 bp fragment. The PCR product was directly cycle-sequenced with the fluorescein-labelled M13 universal sequencing primer (GTAAAACGACGGCCAGT), (Pharmacia Biotech, Uppsala, Sweden) (3 pmol) using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham Life Sciences, Little Chalfont, UK) for 20 PCR cycles (98°C for 30 s, 60°C for 30 s, 72°C for 30 s) in conjunction with the AutoLoad Solid Phase Sequencing Kit (Pharmacia

Biotech), following the manufacturer's instructions and analysed on an automated laser fluorescence (ALF) DNA sequencer (Pharmacia Biotech).

Typing of human leukocyte antigen-DRB1, -DRB3/4/5 and -DQB1 alleles

For high-resolution typing of HLA-DRB1, -DRB3/4/5 and -DQB1, either PCR with sequence-specific primers (PCR-SSP) or sequence-based typing (SBT) was used. First, a low resolution typing was performed by means of group-specific amplification of exon 2 using a 5′ or 3′ specific primer in combination with a generic primer. Depending on the allele groups that were identified, high-resolution typing was performed by a subsequent PCR-SSP using additional primer mixes, or by sequence-based typing [11, 12]. For high-resolution typing of HLA-DPB1, sequence-based typing was used in all instances [13]. High-resolution typing for DRB1/3/4/5, DQB1 and DPB1 was performed for all individuals studied, except in nine cases where high resolution of DRB3/4/5 and/or DQB1 was not possible due to a shortage of material. Since no subtype differences are located in exon 2 of DQB1*02 only low-resolution typing was performed.

The sequence-based typing method for the HLA class II alleles was carried out as described previously [11–13]. For SBT of all class II genes, a PCR product was generated by amplification of exon 2 and, if needed, exon 3 using amplification and sequencing primers located at the 5′ and 3′ end of exon 2 or in introns 1 and 2. Amplification was performed in a final volume of 60 µL consisting of 600 ng DNA, PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$), 5% glycerol, 6 µg cresol red, 200 µM each deoxyribonucleoside triphosphate (dNTP), 20 pmol biotinylated primer, 40 pmol unlabelled primer and 2.0 U Ampli*Taq* DNA polymerase (Perkin-Elmer Corporation). For amplification of DQB1, 1% dimethyl sulphoxide (DMSO) was added to the PCR reaction mixture. PCR amplifications were carried out in a Gene Amp PCR System 9600 (Perkin-Elmer Corporation). The PCR profiles consisted of an initial denaturation at 94°C (2 min), 10 cycles of 10 s at 94°C, 1 min at 65°C and finally 20 cycles (for DRB1/3/4/5 and DQB1) or 30 cycles (for DPB1) of 10 min at 94°C, 50 s at 61°C and 30 s at 72°C, followed by 10 min at 72°C. The sequencing reaction was based on a solid phase approach using attachment of the biotinylated PCR product to streptavidin-coated beads. The sequencing reaction was performed using the Autoread Sequencing Kit (Amersham Pharmacia), according to the suppliers protocol. The sequencing reaction mixture was analysed on a Pharmacia ALFexpress DNA sequencer (Pharmacia Biotech) using a 6% polyacrylamide/7 M urea gel. The gel was run at 1,500 V, 60 mA and 25 W at 5°C for 6 h. After manual evaluation of the processed data, the determination of the HLA subtype was performed with the use of the HLA-Sequityper software (Pharmacia Biotech).

Analysis of the human leukocyte antigen-DPB Glu69 marker

In addition to SBT, genomic DNA samples were screened for the HLA-DPB1*AAG→GAG polymorphism coding the lysine to glutamate 69 amino acid residue using a simple hybridization test designed so that the sensitivity and specificity of each test could be validated by discriminant analysis as already described [6].

Statistical analysis

HLA class II allele and phenotype distributions in berylliosis-affected, Be-sensitized and healthy exposed control groups were compared by means of univariate analysis using odds ratio (OR) and 95% confidence interval (CI); probability values were calculated with the Fisher's exact test. In order to assess the relative strength of the different MHC associations with the disease and sensitization status, and to ascertain possible linkage disequilibria between the HLA-DP and HLA-DR markers and the TNF-α gene, the statistical method described by SVEJGAARD and RYDER [14] was applied.

The method is based on two-by-two tests of the various components of a two-by-four table. Briefly, the frequencies of the phenotypic combinations of the two genetic factors (the DPGlu69, the DRArg74 and the TNF-α-308*2 markers) in patients and controls were calculated ("basic" table). These "basic" data were then analysed by means of various tests, *i.e.* two-by-two tables ("tests" table); for each test an OR was calculated using adequate modifications for "zero" entries and the statistical significance of the OR values was established by Fisher's exact test. Because this analysis involved multiple comparisons, the p-values were corrected; in particular, due to the fact that the association of the DPGlu69 marker was known before the present study, no corrections were done for this marker. On the contrary, p-values for the DRArg74 and the TNF-α-308*2 markers have been corrected for the number of polymorphisms and the number of groups analysed. Due to the fact that no association data were available for the TNF-α-308 polymorphism and berylliosis and no conclusive demonstration of linkage disequilibrium between HLA-DP and TNF-α was found to exist, the other comparisons were corrected for six or by nine, accordingly to the recommendations given by SVEJGAARD and RYDER [14].

In the "tests" table, a series of two-by-two comparisons represents various stratifications of the data for the genetic markers testing (in)dependent contributions to disease susceptibility (table 1, independent association), "interaction" of the factors, difference between the associations (table 1, A association, B association and difference between A and B associations), the value of the combined action of the two factors (table 1, combined association) and linkage disequilibrium of the genetic factors in sensitized/patients and controls (table 1, association between A and B). The tests performed were aimed to

Table 1. – Quantification of the association and interaction levels between the human leukocyte antigen (HLA)-DPB1Glu69 (DPGlu69), the HLA-DRB1/B3Arg74 (DRArg74) and the tumour necrosis factor (TNF)-α-308*2 genetic markers in beryllium-sensitized individuals ("sensitized" subgroup) and in berylliosis patients ("disease case" subgroup)

Factor A cf. Factor B	Independent association		A association		B association		Difference between A and B associations	Combined association	Association between A and B	
	A	B	++ versus -+	+- versus --	++ versus +-	-+ versus --	+- versus -+	++ versus --	"Sensitized"	Controls
"Sensitized" subgroup										
DPGlu69 cf. DRArg74	0.9	3.9#	0.4	2.2	1.3	7.9#	0.3	2.9	0.2	1.4
TNF-α-308*2 cf. DPGlu69	5.0#	0.9	2.5	8.8#	0.4	1.3	6.8#	3.2	0.6	2.2
TNF-α-308*2 cf. DRArg74	5.0#	3.7#	3.2	4.6¶	2.1	3.1	1.5¶	9.9#	2.2	3.2
"Disease case" subgroup										
DPGlu69 cf. DRArg74	3.5¶	0.9	3.3	3.4¶	0.9	0.9	3.7	3.0	1.3	1.4
TNF-α-308*2 cf. DPGlu69	4.6#	3.4¶	3.8	3.7	2.6	2.5	1.5	9.7#	2.3	2.2
TNF-α-308*2 cf. DRArg74	4.6#	0.9	2.9	5.9#	0.5	1.0	6.2¶	2.8	1.6	3.2

Data are presented as odds ratios (ORs). #: p-values are significant (p<0.05) after correction; ¶: p-values <0.05 before, but not after correction. All other figures indicate nonsignificant p-values.

verify various hypotheses and an interpretation is given based upon the significance values. According to SVEJGAARD and RYDER [14], p-values 0.01–0.05 should be regarded as "probably significant" and only p-values <0.01 should be considered as "significant". In fact, the retrospective analysis of HLA-association studies suggests that only these latter p-values have been confirmed in multiple independent studies [14]. However, several authorities in the HLA-disease field [14] strongly recommend the use of phenotypic (or supratypic) markers instead of allelic frequencies for specific reasons: 1) to avoid the bias of increased statistical power determined by the artificial doubling of the population size inherent to allelic frequencies analysis (from N patients to 2×N genes); 2) the phenotype analysis appears to be more appropriate because usually the gene product and not the gene itself is responsible for disease susceptibility or protection [14]. Accordingly, allelic variant analysis data are presented as phenotypic data and the above definitions of "probably significant" and "significant" are used throughout the manuscript.

Results

Human leukocyte antigen-DR, -DQ and -DP marker frequencies

The frequencies of HLA-DRB1*0301, HLA-DRB3*0101, HLA-DQB1*02 and HLA-DPB1*0501 alleles were higher in the "sensitized" subgroup, while HLA-DRB3*0301 was higher and HLA-DRB4*0103 was lower in the "disease case" subgroup (tables 2–5). None of the differences was statistically significant after correction for the number of alleles tested.

The inspection of the polymorphic amino acid residues in the sensitization-associated alleles HLA-DRB1*0301 and HLA-DRB3*0101 showed 82%

Table 2. – Frequencies of the human leukocyte antigen (HLA)-DP alleles# in the study populations

DPB1 alleles	Control	"Sensitized"	"Disease case"
0101#	6 (3.2)	3 (6.5)	2 (4.6)
0201	32 (17.2)	6 (13.0)	12 (27.3)
0202	1 (0.5)		
0301	18 (9.7)	2 (4.4)	4 (9.1)
0401	71 (38.2)	19 (41.3)	10 (22.7)
0402	28 (15.1)	2 (4.4)	4 (9.1)
0501	4 (2.2)	5 (10.9)	1 (2.3)
0601	2 (1.1)	2 (4.4)	1 (2.3)
0901			1 (2.3)
1001	5 (2.7)		3 (6.8)
1101	4 (2.2)	1 (2.2)	1 (2.3)
1301	1 (0.6)	1 (2.2)	
1401	4 (2.2)		1 (2.3)
1501	2 (1.1)	1 (2.2)	
1601		1 (2.2)	1 (2.3)
1701			2 (4.6)
1801	1 (0.6)		
1901	1 (0.6)		1 (2.3)
2001	1 (0.6)	2 (4.4)	
2301	2 (1.1)	1 (2.2)	
3501	1 (0.6)		
7701¶	1 (0.6)		
7801¶	1 (0.6)		

Data are presented as n (%). #: the allele DPB1*0101 includes the allele splits *01011 (controls: five alleles, 2.69% allele frequency) and *01012 (controls: one allele, 0.54% allele frequency) expressing all the same amino acid sequence; ¶: these HLA-DPB1 alleles have been identified in this study population (see [15]).

Table 3. – Frequencies of the human leukocyte antigen (HLA)-DQ alleles in the study populations

DQB1 alleles	Control	"Sensitized"	"Disease case"
02[#]	30 (16.1)	15 (32.6)	5 (11.4)
0201		1 (2.2)	2 (4.5)
0202	1 (0.5)	1 (2.2)	
03[¶]	6 (3.2)	1 (2.2)	
0301	35 (18.8)	6 (13.0)	7 (15.9)
0302	20 (10.7)	3 (6.5)	1 (2.3)
0303	9 (4.8)	1 (2.2)	2 (4.5)
0304	1 (0.5)		
0402	6 (3.2)	1 (2.2)	
05[¶]	1 (0.5)	1 (2.2)	
0501	14 (7.5)	2 (4.4)	5 (11.4)
0502	3 (1.6)	1 (2.2)	1 (2.3)
0503	6 (3.2)	2 (4.4)	1 (2.3)
06[¶]	3 (1.6)	1 (2.2)	2 (4.5)
0601	2 (1.1)		
0602	24 (12.9)	8 (17.4)	7 (15.9)
0603	13 (7.0)		4 (9.1)
0603	1 (0.5)		
0604	9 (4.8)	2 (4.4)	5 (11.4)
0605			2 (4.5)
0609	2 (1.1)		

Data are presented as n (%). [#]: for DQB1*02, no subtype differences are located in exon 2 and therefore only low resolution typing resulting are given; [¶]: for 15 alleles (from nine subjects), high-resolution typing of DQB1 was not possible due to shortage of material, and the data obtained allowed the DQ serotype assignment only.

Table 4. – Frequencies of the human leukocyte antigen (HLA)-DRB1 alleles in the study populations

DRB1 alleles	Control	"Sensitized"	"Disease case"
01[#]		1 (2.2)	
0101	13 (7.0)	2 (4.4)	4 (9.1)
0103	1 (0.5)		1 (2.3)
0301	14 (7.5)	14 (30.4)	4 (9.1)
0302	1 (0.5)		
04[#]	1 (0.5)		
0401	20 (10.7)	1 (2.2)	
0402	1 (0.5)	1 (2.2)	
0404	4 (2.2)	2 (4.4)	1 (2.3)
0405	3 (1.6)	1 (2.2)	
0407	1 (0.5)		
0408	2 (1.1)		
0410	1 (0.5)		
07[#]		1 (2.2)	
0701	24 (12.9)	2 (4.4)	5 (11.4)
0801	4 (2.2)	1 (2.2)	
0806			2 (4.5)
0810		1 (2.2)	
0901		1 (2.2)	
1001	1 (0.5)		
1101	17 (9.1)	2 (4.4)	4 (9.1)
1102	2 (1.1)	1 (2.2)	1 (2.3)
1103	5 (2.7)		1 (2.3)
1104	3 (1.6)		1 (2.3)
1201	1 (0.5)	1 (2.2)	
1301	15 (8.1)	1 (2.2)	6 (13.6)
1302	11 (5.9)	2 (4.4)	7 (15.9)
1303	4 (2.2)		
1401	6 (3.2)	2 (4.4)	1 (2.3)
1406	1 (0.5)		
1501	24 (12.9)	8 (17.4)	5 (11.4)
1502	3 (1.6)		
1601	3 (1.6)	1 (2.2)	1 (2.3)

Data are presented as n (%). [#]: for three alleles (from two subjects), high-resolution typing of DRB1 was not possible due to shortage of material, and the data obtained allowed the DR serotype assignment only.

homology, suggesting that shared polymorphic residues could in fact be associated with sensitization to Be. The comparison between polymorphic residues expressed by the alleles, positively or negatively associated with disease or sensitization, showed that residues DRTyr26 and DRArg74 were unique to sensitization-associated alleles.

Strikingly, the frequency of the DRArg74 marker was significantly higher in the "sensitized" subgroup than in controls (OR 3.96, p=0.005, 95% CI 1.5–10.1). In contrast, in the "disease case" subgroup it was similar to controls (OR 0.89, nonsignificant, 95% CI 0.31–2.6; fig. 1). Also, the DRArg74 marker frequency in the "sensitized" subgroup was significantly higher than in the "disease case" subgroup (χ^2 test, p=0.044).

Consistent with previous study results, the frequency of the DPGlu69 marker was significantly higher in the "disease case" subgroup than in controls (OR 3.7, p=0.016, 95% CI 1.4–10.0; fig. 1). In contrast, its frequency in the "sensitized" subgroup was not different from that in controls (OR 0.89, nonsignificant, 95% CI 0.30–2.22). As expected, the frequencies of several DPGlu69-positive HLA-DPB1 alleles were also increased in the "disease case" subgroup: HLA-DPB1*0201 27.3% (controls: 17.2%), *0601 2.3% (controls: 1.1%), *0901 2.3% (controls: 0%), *1001 6.8% (controls: 2.7%), *1601 2.3% (controls: 0%), *1701 4.6% (controls: 0%), *1901 2.3% (controls: 0.5%). Similarly, the frequencies of the DPGlu69-positive HLA-DPB1 allele phenotypes were also increased in the "disease case" subgroup (table 6). None of these differences, possibly due to the small size of the study group, were statistically significant in comparison to the phenotypic frequencies in the control group.

Table 5. – Frequencies of the human leukocyte antigen (HLA)-DRB3*, 4*, 5* alleles in the study populations

DRB alleles	Control	"Sensitized"	"Disease case"
3*0101	19 (10.3)	16 (34.8)	5 (11.4)
3*0201	3 (1.6)		
3*0202	53 (28.8)	7 (15.2)	16 (36.4)
3*0301	13 (7.1)	2 (4.4)	8 (18.2)
4*[#]	6 (3.3)	2 (4.4)	
4*01[#]	1 (0.5)		
4*0101	14 (7.6)	2 (4.4)	4 (9.1)
4*0103	43 (23.4)	6 (13.0)	3 (6.8)
5*[#]	2 (1.1)		
5*0101	25 (13.6)	10 (21.7)	6 (13.6)
5*0102	2 (1.1)		
5*0202	3 (1.6)	1 (2.2)	2 (4.5)

Data are presented as n (%). [#]: for nine alleles (from seven subjects) high-resolution typing of DRB4* or DRB5* allele was not possible due to shortage of material, and the data obtained allowed the DR serotype assignment only.
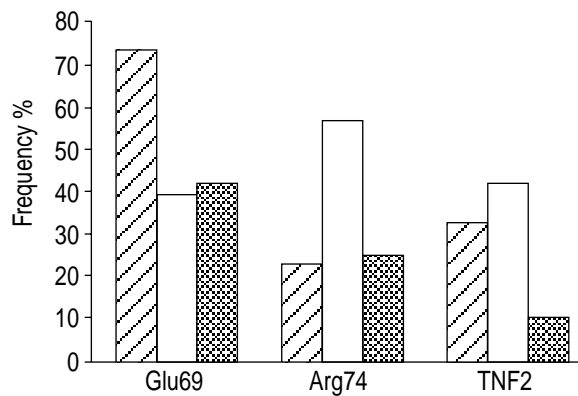
Fig. 1. – Frequencies (%) of the genetic markers tumour necrosis factor (TNF)-α-308*2, human leukocyte antigen (HLA)-DRArg74 and HLA-DPGlu69 in the "disease case" (▨) the "sensitized" (□) and the control (▩) groups.

Tumour necrosis factor-α-308 polymorphism analysis

The frequency of the TNF-α-308*2 allele was significantly increased in the whole BeLPT-positive group compared to BeLPT-negative controls (BeLPT-positive 51.1% *versus* BeLPT-negative 16.1%, OR 7.8, corrected $p<0.0001$, 95% CI 3.2–19.1). When the "sensitized" and the "disease case" subgroups were compared separately to the controls, the frequency of TNF-α-308*2 was significantly higher in both subgroups (fig. 1), and it was similar in the "sensitized" and the "disease case" subgroup (χ^2 test=1.8, nonsignificant; fig. 1).

Analysis for gene interactions

The evaluation of the interaction between the DPGlu69, DRArg74 and TNF-α-308*2 markers, using the analysis of multiple HLA associations with disease described by SVEJGAARD and RYDER [14], showed a positive interaction between TNF-α-308*2

and DRArg74 in the association with the "sensitized" subgroup (table 1, combined association) in the absence of linkage disequilibrium (table 1, association between A and B), but no interaction in the association with the "disease case" subgroup (table 1, combined association). Conversely, while there was no interaction between TNF-α-308*2 and DPGlu69 in the association of the TNF-α-308*2 marker with the "sensitized" subgroup (table 1, combined association) there was a positive interaction in the association with the "disease case" subgroup (table 1, combined association), in the absence of linkage disequilibrium (table 1, association between A and B). In addition, there was an association of DPGlu69 with the "sensitized" DRArg74-negative subjects (table 1, B association -+ *versus* --), but no positive interaction between the two markers either in the "sensitized" or the "disease case" subgroups (table 1, A association ++ *versus* -+).

Discussion

Current concepts of the pathogenesis of berylliosis are that the granulomatous reaction to inhaled Be will develop, after a variable latency period, in the majority, albeit not all, of individuals who develop a T-cell reaction to Be [4]. Previous studies have shown that in individuals with the granulomatous reaction, the reaction to Be is maintained by the activation of CD4+ type-1 T-cells, which are capable of recognizing Be presented by class II MHC molecules [1]. Studies from the authors' laboratory and from others have shown that susceptibility to berylliosis is associated with an HLA-DP marker, the supratypic marker HLA-DPBGlu69 [5–7], although WANG *et al.* [7] have claimed that susceptibility to beryllium disease is associated with rarer HLA-DPB1Glu69-positive alleles through a yet to be defined mechanism. This study shows that the supratypic marker DPGlu69 is associated with the presentation as "disease case"

Table 6. – Frequencies of the Glu69-positive and Glu69-negative phenotypes and of the Glu69-positive human leukocyte antigen (HLA)-DPB1 alleles in the "sensitized" subgroup, the "disease case" subgroup and in controls

Phenotypic frequencies	"Sensitized"	"Disease case"	Control
Patients n	23	22	93
Glu69-negatives	22 (95.7)	17 (77.3)	9 (96.8)
Glu69-positives	9 (39.1)	16 (72.7)[#]	37 (39.8)
Glu69-positive HLA-DPB1 alleles			
HLA-DPB1*0201	5 (21.7)	8 (36.4)	27 (29.0)
HLA-DPB1*0202	0 (0)	0 (0)	1 (1.1)
HLA-DPB1*0601	2 (8.7)	1 (4.5)	2 (2.2)
HLA-DPB1*0901	0 (0)	1 (4.5)	0 (0)
HLA-DPB1*1001	0 (0)	3 (13.6)	5 (5.4)
HLA-DPB1*1301	1 (4.3)	0 (0)	1 (1.1)
HLA-DPB1*1601	1 (4.3)	1 (4.5)	0 (0)
HLA-DPB1*1701	0 (0)	2 (9.1)	0 (0)
HLA-DPB1*1901	0 (0)	1 (4.5)	1 (1.1)

Data are presented as n (%) unless otherwise stated. In this analysis, the number of subjects carrying Glu69-positive phenotypes (Glu69-positives) does not correspond to the number of subjects carrying Glu69-positive HLA-DPB1 alleles in the same column. Subjects homozygous for Glu69, due to the carriage of two different Glu69-positive HLA-DPB1 alleles, are listed once in the "Glu69-positives" row and twice in the Glu69-positive HLA-DPB1 alleles rows, once for each of the alleles carried. [#]: p=0.023 *versus* "sensitized" and p=0.05 *versus* controls.

(*i.e.* with a profusion of lung granulomas sufficient for TBB detection of the pathological alteration), but it is not associated with the presentation as "sensitized" (*i.e.* with undetectable lung granulomas), which instead appears to be associated with other MHC class II markers, possibly the supratypic marker DRArg74. Finally, both "sensitization" and "disease case" are strongly associated with the MHC locus cytokine gene TNF-α, TNF-α-308*2 marker.

The association of different HLA markers with disease presentation is reminiscent of a number of immunogenetic observations. In leprosy, MT1, MB1 and DC1 are associated with lepromatous leprosy [15], while DR3 and the amino acid residues DRB1Arg13, 70 and 71 are associated with tuberculoid leprosy [16]. In sarcoidosis, DR3 is associated with presentation as spontaneously resolving stage I disease, while DR4 and DR5 are associated with presentation as progressive stage III disease [9]. In schistosomiasis japonica, Dw19, DRw13 and DQw1 are associated with the presence of liver fibrosis, and Dw12, DR2, DQw1, DPA1*0301 and DPB1*0201 with its absence [17, 18].

Current concepts of HLA disease association are that the amino acid residues implicated in peptide binding to HLA-DR molecules for presentation to the T-cell antigen receptor [19] are also associated with several immune disorders as susceptibility factors [14]. In their recent study, WANG *et al.* [7] found a significant increase in the frequency of the rare alleles HLA-DPB1*1701, *0901 and *1001, along with a decrease of the common allele HLA-DPB1*0201 in an American population of beryllium disease patients and suggested that these, and not the supratypic marker that they shared, represented the disease marker. In the present study, increased frequencies of Glu69 HLA-DPB1 alleles, including HLA-DPB1*0201, were also found both in the "sensitized" and the "disease case" groups. However, the demonstration by LOMBARDI *et al.* [8] that the Glu69 residue of the HLA-DP β chain is directly involved in presentation of Be to the T-cells of HLA-DPBGlu69 subjects with berylliosis, consistent with the observation that the HLA-DPGlu69 residue is important in autoimmunity and alloreactivity [20] and with the finding of oligoclonal beryllium-reactive T-cells [21], strongly indicates that the sequence coding for the HLA-DPB1 amino acid residue Glu69 is not only a supratypic marker but it is the immune response gene of berylliosis. Interestingly, the observation that HLA-DPGlu69 is associated with susceptibility to hard metal lung disease and that cobalt directly interacts with the HLA-DPB1Glu69 molecule [22] suggests that this HLA-DP molecule is endowed with the ability of interacting with certain metal cations, possibly through direct binding.

The DRArg74 residue, which is involved in peptide binding by HLA-DR molecules [19], may also play a direct role in the immune response to Be. However, no evidence is yet available for a role of HLA-DRB3 molecules in Be presentation. On the other hand, in the context that interferon gamma (IFN-γ) plays a pivotal role in granuloma formation and is overexpressed in response to Be *in vitro* [23], the apparent

"protective" effect of DRArg74 on disease pathogenesis may be due to association of the HLA-DR3 alleles (*i.e.* DRArg74) with low production of IFN-γ [24]. Since the authors only assessed the association of DRArg74 with TNF-α-308*2 and DPGlu69, a linkage of DRArg74 with low IFN-γ-stimulated production cannot be ruled out.

With regard to the TNF-α-308*2 marker, it is known that TNF-308*2 is strongly associated with the high TNF-α production in subjects carrying the HLA-A1, -B8 and -DR3 haplotype. Current concepts are that elevated TNF-α production associated with the -308 polymorphism may alter the course of an immune response, thus increasing the risk of disease development [25]. This marker has been associated with susceptibility to sarcoidosis [26] and silicosis [27]. It may be hypothesized that TNF-α plays an initiating role in Be sensitization since it is known that TNF-α messenger ribonucleic acid (mRNA) and protein expression are increased upon Be stimulation of the H36.12j mouse macrophage cell line [28], a process similar to experimental nickel-contact dermatitis where nickel-upregulated keratinocyte TNF-α production plays a role in skin sensitization [29].

In summary, the present study confirms that the human leukocyte antigen-DPGlu69 marker is associated with beryllium disease and identifies another gene marker, tumour necrosis factor-α-308*2, also associated with disease risk. In addition, it suggests that more than one major histocompatibility complex gene may be involved in the determination of disease susceptibility, playing different and even contrasting roles in disease pathogenesis. In the context that genetic screening for disease risk could be cost-effective [30], the characterization of disease-associated genes may lead to the identification of reliable markers of disease risk for the prevention of berylliosis.

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