HLA class II DPB1 and DRB1 polymorphisms associated with genetic susceptibility to beryllium toxicity

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ABSTRACT

Objectives Chronic beryllium disease (CBD) is a hypersensitivity granulomatous pulmonary disease caused by exposure to the metal beryllium (Be^{2+}). Our objective was to extend current knowledge of the genetics of beryllium disease by examining all HLA-DPB1 and HLA-DPR1 gene polymorphisms and the interactions between them.

Methods DNA-based typing of HLA-DPB1 and HLA-DRB1 loci at the allele level was performed on 65 CBD, 44 beryllium sensitised (BeS) but without CBD and 288 non-affected, beryllium exposed controls.

Results The DPβE69 residue regardless of zygosity, but particularly if present on non-*0201 alleles, was of primary importance for the development of CBD and BeS, while other negatively charged residues DPβDE55, 56 and DPβDE84, 85 incrementally increased, although not independently, the risk. The DPβE69 positive alleles with charge −7 or −9 were associated with both CBD and BeS. The polymorphic residues DPβE69, DPβDE55, 56 and DPβDE84, 85 were responsible for the −9 charge and the first two residues for the −7 charge.

Conclusions In the absence of DPβE69, DRβE71 is a risk factor for CBD and BeS. DPβE69 and DRβE71 are adjacent to other amino acids that are also negatively charged, suggesting that the positively charged Be^{2+} modifies the local environment of the epitopes in a way that promotes interactions between peptides and T cells and results in CBD. Finally, the protective effect of the DPB1*0201 positive haplotype may involve particular polymorphisms outside of the DPB1 gene.

What this paper adds

- This study confirmed that individuals with glutamic acid at position 69 on the HLA-DPβ chain are at increased susceptibility for developing chronic beryllium disease (CBD) and/or beryllium sensitisation (BeS).
- However, not all individuals with CBD or BeS are HLA-DPβ69 positive and 30–40% of beryllium exposed workers are HLA-DPβ 69 positive but do not develop CBD or BeS.
- This study identified certain DPβE69 alleles (DPB1*0201 negative alleles) and polymorphisms of the HLA-DRβ chain, particularly DRβE71, that were important in addition to DPβE69 in explaining genetic susceptibility to the development of CBD and/or BeS.
- This study found that the total negative charge contributed by specific polymorphisms within the DPβ chain are associated with CBD and BeS but only in conjunction with DPβE69.
- The genetic polymorphisms identified in our study appear to be functionally important in how peptides are presented to T cells and therefore are directly involved in the pathophysiology of CBD and/or BeS.

INTRODUCTION

Workers exposed to beryllium have developed both sensitisation (BeS) and chronic beryllium disease (CBD).[1] As early as the 1950s it was hypothesised that the disease was immunologically mediated after it was observed that CBD occurred after both high and low levels of exposure in a small percentage of workers.[2] Genetic susceptibility to the development of CBD was reported in 1993[3] and to BeS in 2001[4] and confirmed and refined by subsequent investigators[5–10] among individuals with glutamic acid at position 69 on the HLA-DPβ chain. Studies with blocking monoclonal antibodies have strongly suggested the functional significance of this polymorphism.[11–14] The hypothesised mechanism is that beryllium is a positive bivalent cation which may influence peptide binding directly or indirectly to the HLA molecules.[15] This influence may occur by changing the local electrostatic environment and affecting peptide binding to the groove of the HLA class II molecules,[14] which in sequence may also change the interaction of the T cell receptor of CD4+ T cells with the HLA–peptide complex. The HLA system has a major role in the adoptive immune response. Understanding the change initiated by beryllium in those who are genetically susceptible will not only help to better control beryllium toxicity but also is important to understanding other immunologically mediated diseases.

Previous studies have found that 62–97% of individuals with CBD or BeS are HLA-DPβE69 positive but that 30–40% of beryllium exposed workers who do not develop CBD or BeS are also positive. Thus, other genetic and/or exposure metrics play a role in determining which individuals develop CBD and BeS. Investigators have examined whether individuals who were homozygous for DPβE69 containing alleles[6 7 10] or who had particular alleles containing DPβE69 (ie, DPB1*0201 negative alleles) were at higher risk for developing CBD.[16] The other class II genes of the HLA complex that have received a lot of attention

in relation to the risk for the development of beryllium disease are the genes of the DR family.[16] Among DPβE69 negative subjects, findings have been reported related to the HLA-DRB1 gene, such as the association of DRβF47 with beryllium hypersensitivity (CBD and BeS together)[17] and the association of DRB1*1301 with CBD.[6] Bill *et al*[11] found that fibroblasts expressing mutated DP2 molecules at position 69 (E to K) and also fibroblasts expressing DR13 molecules at position 71 (E to R) were capable of inducing beryllium specific proliferation and INF-γ expression by lung CD4+ T cells. Findings that suggest that there may be other HLA-related markers besides the DPB1 and DRB1 genes that influence the development of BeS or CBD has led to investigations of other genetic markers, including butyrophilin-like 2 (BTNL2) polymorphisms,[18] transforming growth factor β (TGF-β1) polymorphisms[19 20] and tumour necrosis factor (TNF) polymorphisms.[21 22]

This manuscript expands on previous research on the genetics of beryllium by examining all HLA-DPB1 and HLA-DPR1 gene polymorphisms and interactions between them in a cohort with a high prevalence of CBD and BeS.[23] Preliminary results of the genetic analysis were presented at the 2007 American Thoracic Society International Conference.[24]

METHODS

A total of 5490 workers were on the payroll for 2 or more days at one of two beryllium production facilities in eastern Pennsylvania. Facility A was open from 1958 to 1978 and employed 1349 individuals during that time and facility B was open from 1935 to 2000 and employed 4141 individuals during that time. Using various databases it was determined, that as of the end of 1988, 328 (24.3%) individuals from facility A and 2293 (55.4%) individuals from facility B had died. The first mailings to members of the cohort not identified to have died in 1988 began in 1996. Follow-up calls were conducted until the individual declined or agreed to participate. In 1996, 148 (11%) individuals from facility A and 177 (4.3%) individuals from facility B could not be located. Among the 873 workers from facility A who were reached and invited to participate in a medical screening, 160 said they worked for the company but did not work at the beryllium production facility, 65 declined to participate, 86 completed a questionnaire only and 562 participated in the medical screening. Among the 1671 workers from facility B contacted about the medical screening, 35 said they worked for the company but not at the beryllium production facility, 191 declined to participate, 474 completed a questionnaire only and 971 participated in the medical screening. Accordingly, a total of 1533 individuals participated in the medical screening, 256 declined and 560 completed a questionnaire only. The medical screening occurred from 1996 through 2001.

None of the subjects in this cohort were included in previous publications of genetic testing including those where there were overlapping authors.[5 17]

Any individual with two positive beryllium lymphocyte proliferation tests (LPTs) and/or a chest radiograph reading of ≥1/0 in parenchymal profusion by at least two of three B readers (physicians certified to interpret chest radiographs for pneumoconiosis) was referred for bronchoscopy, the testing of lavage fluid for beryllium lymphocyte proliferation and a transbronchial biopsy. The BeLPT was considered positive when the proliferation of blood lymphocytes to three different concentrations of $BeSO_4$ at 5 and 7 days when both the stimulation index (SI) (the ratio of the counts per minutes (CPMS) of the stimulated cells to the CPMS of the unstimulated cells) was greater than normal and the result was statistically significant (the standardised log of the SI was greater than 3.1). The individuals eligible for genetic testing met the definitions for CBD (which was non-caseating granuloma on lung biopsy and positive lavage or two positive blood LPTs), probable CBD (which was a positive lavage LPT or positive radiograph and two positive blood LPTs) or BeS (which was two positive blood LPTs) or were controls with completely normal results (table 1). Individuals meeting the definitions for CBD or probable CBD were combined in the analysis as the CBD group. Twenty of our CBD cases had not had lung biopsies and met the criteria for CBD based on their lavage or radiographic findings (table 1). Two controls, matched by facility, gender and year of birth within 3 years whose results on chest radiograph and LPT testing were totally normal, were selected for each individual with CBD or sensitisation. A total of 288 of the 1299 individuals who participated in the medical screening and had completely normal testing for CBD and/or sensitisation, were selected as controls and had genetic testing. Another 99 individuals had some abnormality but did not meet the definition for CBD or BeS and were not included in the potential group of controls.

Among the individuals who participated in the medical screening, 80 met the definition for CBD and 55 for BeS. Fifteen individuals with CBD or probable CBD and 11 with BeS were not tested because they did not provide either consent or blood for the genetic testing. These 26 individuals did not differ by age, gender or race from those who had genetic testing.

Genomic DNA was prepared 4–10 years after collection using Qiagen columns (QiaAmp 96 DNA Blood kit; Qiagen, Valencia, California, USA) from a venous whole blood sample that had been frozen the day after collection. The DPB1 gene (exon 2 and 3) was characterised with high resolution typing using the PCR-SSP method (Pel-Freez Clinical Systems, Brown Deer, Wisconsin, USA). For ambiguities or inconsistent patterns of primer amplifications, sequence-based typing was performed and confirmed by bi-directional sequencing-based typing of exon 2

Table 1 Minimum criteria for beryllium disease categories

Disease category	Number of subjects	Bronchial lavage	Biopsy granuloma	Chest radiograph	Beryllium LPT
CBD	37	+BeLPT	Positive	*	*
	8	Negative or not done	Positive	*	Two +BeLPTs
Probable CBD	7	+BeLPT	Not done	*	*
	13	Negative or not done	Not done	Fibrosis any zone	Two +BeLPTs
Possible CBD	18	Not done	Not done	Fibrosis any zone	−BeLPT or single +BeLPT and no re-test
Sensitisation	44	Not done	Negative or not done	Normal	Two +BeLPTs
Possible sensitisation	81	Negative or not done	Negative or not done	Normal	Single +BeLPT and no retest or −BeLPT retest

*Test result does not affect disease categorisation.
BE, beryllium; CBD, chronic beryllium disease; LPT, lymphocyte proliferation test.

(AlleleSEQR HLA-DPB1 SBT kit; Atria Genetics, South San Francisco, California USA). A new allele was identified and was named DPB1*1902.[25] DRB1 typing was performed by sequence-based typing. All genetic analyses were performed without knowledge of whether the sample was from a CBD, BeS or control participant.

Pearson's χ^2 tests or Fisher's exact test and logistic regression were used to compare the frequency of genetic markers between the groups. The false discovery rate was used to indicate statistical significance[26] except when examining a priori associations. Multiple tests regarding the interactive relationship among the different HLA residues, homozygosity and linkage disequilibrium were evaluated by the method of Svejgaard and Ryder.[27 28] The calculations and analyses of negative charge of different HLA-DPB1 alleles positive for the DPβE69 residue were performed as described by Snyder *et al*.[29] The Hardy–Weinberg equilibrium (HWE) was used for the analysis of homozygosity and heterozygosity.

This study was approved by the Michigan State Human Subject Review Board, and separate consent forms were used for medical screening and genetic testing.

RESULTS

Sixty-five individuals with CBD, 44 with BeS and 288 controls had genetic testing.

Average age, gender, race and average exposure were similar in the three groups. The BeS group was more likely to have worked in facility A and to have had shorter duration of work (table 2). The numbers and frequency of individuals (phenotypic frequency) positive for each of the different DPB1 and DRB1 alleles in control, BeS and CBD cases are shown in the online supplement.

The prevalence of individuals positive for the HLA-DPβE69 residue was 60 of 65 (92.3%) among those with CBD (OR 19.14 (95% CI 7.10 to 55.92) $p=1.8\times10^{-16}$), 35 of 44 (79.5%) among those with BeS (OR 6.20 (95% CI 2.73 to 14.47) $p=3.82\times10^{-7}$) and 111 of 288 (38.5%) among controls. The respective attributable risk per cent was 95% and 84%. There was no significant difference between CBD and BeS subjects in the prevalence of homozygotes and heterozygotes for HLA-DPβE69, but both were significantly different compared to controls (figure 1). HWE analysis found that the frequencies of DPβE69 homozygous and heterozygous subjects within the control and the BeS populations were in equilibrium, but in the CBD population the expected number of homozygous subjects was 19, while the observed was 10, and the expected number of heterozygous subjects was 32, while the observed was 50 (p=0.0175).

The distribution of the non-DPB1*0201 alleles among DPβE69 positive subjects was statistically increased in the CBD subjects but not in the BeS subjects (CBD subjects 29 of 60 (48.3%); BeS subjects 16 of 44 (45.7%); controls 36 of 111 (32.4%); CBD OR 1.95 (95% CI 0.97 to 3.91) p=0.041 and BeS OR 1.19 (95% CI 0.54 to 2.63) p=0.64). Among the 20 non-0201 alleles containing HLA-DPβE69 in our study group, 10 were more frequent in individuals with CBD (*0202, *0301, *0601, *0901, *1001, *1101, *1401,*1601, *1701 and *7101) and 10 were less frequent (*0101, *0401, *0501, *1301,*1901, *2001, *2301, *2901, *4501 and *4601).

Table 2 Demographic characteristics of participants

Characteristic	Chronic beryllium disease n=65	Beryllium sensitisation n=44	Controls n=288
Age, years	59.9±9.9	58.5±13.2	59.7±11.2
Male sex, %	94	91	92
Caucasian, %	100	98	98
Facility A, %	49	57	52
Average duration of work, years	9.5±12.7	4.2±6.1	11.2±13.5
Average exposure, μg/m^3	8.2±21.9	7.6±16.7	7.3±16.4

Data are presented as mean±SD.

The distribution of DPβDE 55, 56 containing alleles in CBD subjects was significantly different compared to controls (CBD: 60 of 65 (92.3%); controls: 177 of 288 (61.5%); OR 7.53 (95% CI 2.79 to 21.99) $p=3.3\times10^{-7}$) and BeS subjects (BeS: 37 of 44 (84.1%); OR 3.31 (95% CI 1.36 to 8.46) p=0.004). However, DPβE69 and DPβDE55, 56 were in strong statistically significant linkage disequilibrium in the CBD, BeS and control groups and DPβE69 was statistically significantly independent from DPβDE 55, 56 in CBD and BeS. The combined presence of these epitopes versus their combined absence had a statistically significant effect in both CBD and BeS.

In addition to DPβDE55, 56, DPβV8, DPβH9, DPβL11, DPβV36, DPβD57 and DPβDEAV 84–87 were significantly positively associated with CBD, while DPβA55, DPβA56 and DPβK69 were significantly negatively associated with CBD. DPβH9, DPβV36 and DPβDEAV84–87 were also significantly positively associated with BeS. DPβL11 and DPβV36 behaved like DPβDE55, 56; they were in linkage disequilibrium with DPβE69 and they did not have a significant independent contribution among DPβE69 subjects for both CBD and BeS individuals. DPβV8, DPβD57 and DPβDEAV84–87 were not in linkage disequilibrium with DPβE69 and they did not have an significant independent contribution among DPβE69 subjects, while DPβE69 did have a significant contribution in the presence of any of these residues. This observation was true for both CBD and BeS groups.

In contrast to the other residues, DPβH9 presented a different picture. Among CBD subjects, DPβH9 and DPβE69 were in significant linkage disequilibrium, but while DPβH9 had an significant independent contribution among E69 positive subjects, DPβE69 did not have a significant independent contribution among DPβH9 positive subjects. Among BeS subjects, DPβH9 and DPβE69 were in significant linkage disequilibrium and neither had a significant independent contribution in the presence of the other. When the same residues were examined for their differential distribution among CBD and BeS subjects, there were no statistically significant differences between the two groups.

The above findings indicate that the primary association was with DPβE69 for both CBD and BeS and that the other residues found to be associated did not by themselves confer an additional risk beyond and above that of the DPβE69 residue. The importance of the DPβH9 residue for CBD but not for BeS will have to be re-evaluated upon examination of a different and/or larger data set. Among DPβE69 positive, DPB1*0201 negative CBD but not BeS subjects, the epitope DPβDE55, 56 occurred more often (27 of 29 (93.1%)) than among DPβE69 positive, DPB1*0201 negative controls (18 of 36 (50.0%); OR 13.50 (95% CI 2.51 to 96.16) $p=1.53\times10^{-4}$) as did DPβV36 and DPβI76, which were negatively associated. DPβDE55, 56 and DPβV36 were in strong linkage disequilibrium and neither had a significant independent effect in the presence or absence of the other. No significant differential distributions of any DPβ or DRβ residues were identified among all DPβE69 combined or DPB1*0201 positive only subjects.

HLA-DPB1 alleles positive for a DPβE69 residue that also included 9 or 7 negatively charged amino acids were significantly

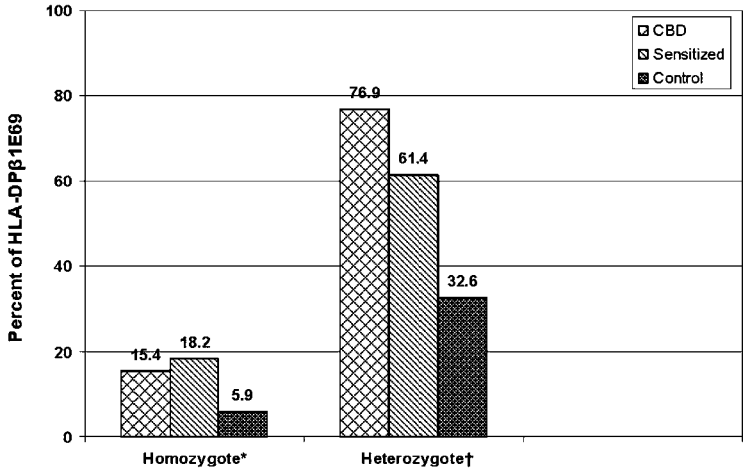

Figure 1 Prevalence of HLA-DPβE69 by beryllium disease category and zygosity.

Number of Cases: Homozygote: CBD=10, BeS=8, Control=17

Heterozygote: CBD=50, BeS=27, Control=94

* CBD vs. control, O.R. 2.90 (95% C.I. 1.16-7.14), p=0.009; BeS vs. control, O.R. 3.54 (95% C.I. 1.29-9.51), p=0.004; CBD vs. BeS, O.R. 0.82 (95% C.I. (0.26-2.55), p=0.70

† CBD vs. control, O.R. 6.88 (95% C.I. 3.53-13.55), p=10^{-7}; BeS vs. control, O.R. 3.28 (95% C.I. 1.63-6.64), p=0.0002; CBD vs. BeS, O.R. 2.10 (95% C.I. 0.84-5.28), p=0.08

associated with CBD and BeS (figure 2). No statistical differences were found for total negative charge categorised as <−13, versus −13 to <−16, versus −16 to −18 (CBD vs control, χ^2=0.22, p=0.895; BeS vs control, χ^2=2.11, p=0.348; CBD vs BeS, χ^2=1.45, p=0.484).

Among the DPβE69 negative individuals with CBD or BeS, the DRβE71 residue was significantly increased: five of five (100%) CBD subjects versus 34 of 177 (19.2%) controls; and nine of nine (100%) BeS subjects versus 34 of 177 (19.2%) controls (table 3).

Among DPβE69 negative CBD subjects, five of five (100%) were positive for DRβN37 versus 56 of 177 (31.6%) controls (table 3), but this difference was not significant. The DRβN37 residue was significantly increased when comparing DPβE69 negative BeS to controls (9 of 9 (100%) BeS subjects vs 56 of 177 (31.6%) controls).

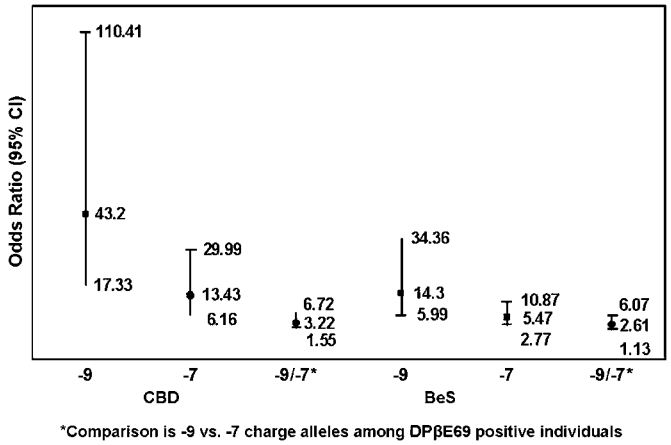

Figure 2 ORs of alleles with −9 or −7 charge among individuals with chronic beryllium disease (CBD) or beryllium sensitisation (BeS) who are DPβE69 positive versus controls who are DPβE69 positive.

Similarly among DPβE69 negative CBD subjects, five of five (100%) were positive and among controls 61 of 177 (34.5%) were positive for the DRβH32 residue. This difference was not significant. However, the DRβH32 residue was significantly differentially distributed between DPβE69 negative BeS and controls (9 of 9 (100%) BeS subjects vs 66 of 177 (37.3%) controls) (table 3).

Residues E71, N37 and H32 in pairs, were in linkage disequilibrium in both BeS and controls. Neither E71, N37 nor H32 were distributed differently among individuals stratified for any of the three amino acids.

Among DPβE69 negative BeS subjects, DRβS11 and DRβS13 were not statistically increased among BeS subjects. Both residues were in strong linkage disequilibrium with DRβE71, in both control and BeS subjects. When the population of alleles was stratified for DRβS11 or DRβS13, the distribution of DRβE71 was significantly increased among BeS subjects as compared to controls (DRβS11: OR 10.54 (95% CI 2.43 to 52.30) p=0.0031; DRβS13: OR 9.00 (95% CI 2.07 to 44.75) p=0.004). When the population of alleles was stratified for DRβE71, neither the DRβS11 nor the DRβS13 residues demonstrated any differential distributions. Table 3 summarises all the DRB1 residues previously reported as being associated with CBD or BeS. We found no association for arginine 74, phenylalanine 47 or tyrosine 26 whether corrected or uncorrected.

Evaluation of the whole cohort for the distribution of DPβE69 and/or DRβ71 found that 65 of 65 (100%) CBD subjects, 44 of 44 (100%) BeS subjects and 145 of 288 (50.3%) controls were positive for either of these amino acids. These associations for both CBD and BeS were highly significant: p=7.1×10^{-14} and p=2.37×10^{-11}, respectively (OR and CI undefined as one cell is zero).

DISCUSSION

Our cohort is one of the largest cohorts of CBD and BeS cases involving the complete typing of DRB1 and DPB1 genes of the

Table 3 Results of analyses of HLA-DRβ with DPβ by glutamic status at position 69 and disease status for DRβ residues previously reported in the medical literature

	DPβE69+		DPβE69−		
DRβ residues	CBD n=60	BeS n=35	CBD n=5	BeS n=9	Previous findings from the medical literature
Arginine 74	NA	NA	NA	NA	E69− only, CBD and BeS combined[17]
Asparagine 37	NA (−)	NA	NA (+)	+	E69− only, CBD and BeS combined[17] E69+ and E69−, CBD and BeS combined[5]
Glutamate 71	NA	NA	+	+	E69− only, CBD[13] E69+ and E69−, CBD and BeS combined[5]
Histidine 32	NA (−)	NA	NA	+	E69− only, CBD and BeS combined[17]
Phenylalanine 47	NA	NA	NA	NA	E69− only, CBD and BeS combined[17]
Serine 11	NA	NA	NA	NA (+)	E69+ and E69−, CBD and BeS combined[5]
Serine 13	NA	NA	NA	NA (+)	E69− only, CBD and BeS combined[17]
Tyrosine 26	NA	NA	NA	NA	E69− only, CBD and BeS combined[17]

+, positive statistical association ($p<0.05$); −, negative statistical association ($p<0.05$); NA, no association; NA (+/−), no statistical association after correction but trend in the direction shown in parentheses.
BeS, beryllium sensitised; CBD, chronic beryllium disease.

HLA class II molecules, including the comprehensive examination of the role of every single polymorphic residue in both isotypes, linkage disequilibrium between the residues as well as the role of homozygosity and negative charges. Members of our cohort had longer latency periods from first and last exposure to beryllium than most previously reported cohorts.

We confirmed the previously reported association of HLA-DPβE69 residue with both CBD (OR 19.14 (95% CI 7.10 to 55.92)) and BeS (OR 6.20 (95% CI 2.73 to 14.47)) with attributable risks of 95% and 84%, respectively.[5–12] Differences in ORs for CBD and BeS reported between studies in the literature probably reflect study differences in confirming CBD (if confirmation rates are low, then individuals who truly have CBD are more likely to be classified as BeS only), latency since first or last exposure to beryllium (in studies with longer latency periods individuals will have had additional time to progress to CBD from BeS) and sample size.

We found, as did previous investigators, that homozygosity for DPβE69 is increased among CBD[6 7 10] and BeS[6 7] subjects in reference to the control group but not for CBD versus BeS[6 7] (figure 1). The increased numbers of homozygous DPβE69 alleles observed in CBD populations was not in excess of that expected by HWE and therefore was a reflection of the increased DPβE69 positive alleles observed in CBD populations and not because homozygosity was a risk factor for the development of CBD.

Like previous investigators, we found that the less common DPβE69 positive, DPB1*0201 negative alleles were more common in CBD individuals than among controls.[10] New results from our study potentially explain this association. Only the DPβDE55, 56 epitope and no other residue was associated with CBD individuals who were DPβE69 positive and DPB1*0201 negative. Since the DPB1*0201 allele is also positive for the DPβDE55, 56 epitope, we hypothesise that other genes on the DPB1*0201 haplotype or protective polymorphisms outside of the DPB1 gene have a protective role that causes DPβDE55, 56 to be not associated with CBD among DPB1*0201 positive subjects.

In another approach to assess the association of different DPβE69 positive alleles with CBD and BeS, Snyder *et al* found an increase in alleles with a −9 charge as compared to alleles with a −7 charge among subjects with CBD but not subjects with BeS and an increase in the total negative charge in CBD versus controls and CBD versus BeS.[29] We found that among the DPβE69 positive alleles with a −7 charge, the amino acids at positions DPβ55 and DPβ56, were D and E, respectively (except for the allele DPB1*1301 that was DPβAA55, 56, DPβDE84, 85) and that all the −9 charge positive alleles were DPβE69, DPβDE55, 56 and DPβDE84, 85 positive. DPβE69 and DPβDE55, 56 (−7 charge) increase the risk of developing CBD and BeS, and DPβE69, DPβDE55, 56 and DPβDE84, 85 (−9 charge) confer an even greater risk of developing CBD and BeS (figure 2). The residues DPβDE55, 56 and DPβDE84, 85, which were in linkage disequilibrium with DPβE69 did not confer a risk independent of DPβE69 except for the increased risk for CBD in individuals with the DPβDE55, 56 epitope and DPB1*0201 negative haplotypes.

In addition to the variable positions at DPβE69, DPβDE55, 56 and DPβDE84, 85, DPβE67, DPβE68 and DPβE82 are conserved and are acidic residues, so when an allele is DPβE69 or DPβDE84, 85 positive, the DPβEEE67–69 or DPβELDE82–85 epitope is characterised by a triple negative charge. Furthermore, the DPβ57, although polymorphic, is almost always negatively charged (D and E), so when DPβ55, 56 is negatively charged there is another negatively charged triplet in the β chain. The DPβ67–69 epitope is in the middle and the top of the β chain and the other two epitopes, the DPβ55–57 and the DPβ82–85, are located at the two ends of the β chain, influencing the opening of the cleft. Besides these linear sequences, the spatial configuration brings DPβ26 and 69 and perhaps other negative charged residues into close proximity. When beryllium interacts with these residues, possibly by forming a tetrahedron with adjacent DPβE69, DPβE26 residues and two molecules of water,[29] it neutralises the negative charges and facilitates peptide–DP complexes which stimulate specific T cell responses. Amicosante *et al* have hypothesised that Be^{2+} presentation may be mediated by selected peptides expressing amino acid residues with high affinity for pocket 4 of the DP molecule and with high electron donor capability.[15] Future crystallographic data involving Be^{2+} and the peptide–MHC complex and evaluation of the binding of peptide and T cell responses in the presence or absence of beryllium are needed to explain how DPβE69, DPβDE55, 56 and DPβDE84, 85 epitopes interact. That the DPβDE55, 56 epitope may have an effect on the binding of beryllium or peptide is consistent with data showing the location of the DPβD55 is equivalent to D57 of the DQβ chain, which is associated with type 1 diabetes[30] and the DPβDE55, 56 epitope is equivalent to the DQβRLD55–57 epitope, which is associated with acute inflammatory demyelinating polyneuropathy.[31]

Similarly to previous investigators, we found that a small percentage of individuals with CBD or BeS were HLA-DPβE69 negative (7.7% and 20.5%, respectively).[16] Inhibition of beryllium stimulated T cell proliferation assays by anti-DR monoclonal

antibodies in DPβE69 negative subjects argues for a role of the DR molecule in the development of CBD and BeS.[17]

New findings in our analysis show that DRβE71 was significantly increased in both CBD and BeS subjects compared to controls, while DRβN37 and DRβH32 were significantly increased among BeS but not among CBD subjects. However, since a small number of DPβE69 negative subjects limited our statistical power, and we used a statistical correction factor because of multiple comparisons, it is possible that DRβN37 and DRβH32 also play a role in CBD. When these three residues were evaluated for strength of association in the BeS group, they were all in strong linkage disequilibrium among themselves and none were more strongly associated with BeS than the other.

DRβS11 and DRβS13 have been previously suggested to be associated with BeS and CBD. We found an association among BeS but not CBD subjects. Peptide binding studies and T cell functional assays are needed to evaluate if beryllium-influenced peptide binding patterns are different for DR molecules and may affect the ensuing T cell responses such that they only lead to BeS and not CBD.

Other investigators have attributed their findings of an association of DRβN37 and DRβH32 with a combined group of CBD and BeS DPβE69 negative subjects to linkage disequilibrium with DRβF47 and not to a direct role of DRβN37.[17] We did not find an association with DRβF47 and no linkage disequilibrium was observed between DRβF47 and DRβN37 or between DRβF47 and DRβH32. In our study, in the absence of the DPβE69 residue, only the DRB1 locus with the polymorphism that corresponds to DRβE71 was significant for both CBD and BeS.

The significance of DRβE71 has been demonstrated in functional assays where DR molecules with E71 induced beryllium specific proliferation and IFN-γ expression by lung CD4 positive T cells[9] and the association is consistent with previous studies that have shown that the DRβ71 position in pocket 4 of the HLA molecule influences the charge of the P4 pocket[32] and is critical for both peptide binding and T cell interactions in pemphigus vulgaris,[33] rheumatoid arthritis,[34 35] multiple sclerosis,[36–38] tuberculoid leprosy[39] and epithelial ovarian carcinoma.[40]

All CBD and BeS subjects were positive for DPβE69 and/or DRβE71, but only about 50% of the control subjects were positive for either of the two residues. Although these HLA polymorphisms appear necessary for developing CBD and/or BeS, the fact that there are controls exposed to beryllium without sensitisation or disease indicates that additional factors contribute to disease outcomes. Current factors being studied include other yet to be identified genetic polymorphisms, and cumulative, peak or type of exposure (ie, particle size or physical property) that may interact with genetic variability.[8 41 42] Further analysis with stratification based on the specific HLA polymorphisms should reveal differences in genome-wide association studies that will go beyond HLA polymorphisms. In our previous publication we were unable to show an exposure–response relationship for CBD or BeS for facility A, the smaller of the two facilities.[23] The results presented here may assist in refining exposure metrics potentially related to gene–environment interactions at the HLA-DPβ1 and HLA-DRβ1 loci.

One limitation of our study was the statistical power when we stratified by DPβE69 negative CBD or BeS subjects, with five and nine subjects respectively, and our use of conservative correction factors. Most of the comparisons had power <0.50. Although comparing CBD or BeS to control, the comparisons of either zygosity for DPβE69, non-0201 alleles in DPβE69 positive individuals, DRβN37 in DPβE69 negative individuals, and DRβH32 in E69 negative individuals had power >0.50. A second limitation was that only 55% of the individuals with two positive BeLPT test results and a normal chest radiograph agreed to have bronchoscopy. Based on the results of those who had bronchoscopy, one would expect up to 48% of the 44 individuals who we classified as BeS would have had granuloma and therefore should have really been classified as CBD.[23] Inversely, one could argue that some of the probable CBDs (ie, positive lavage but normal chest film or abnormal chest film and sensitisation but subject never biopsied to determine if they had granuloma) were not CBD but rather sensitisation only. This potential misclassification of BeS and CBD would reduce the likelihood of seeing differences between the CBD and BeS subjects but not in comparison to the control group. Despite studies that have reported on false negative rates for BeLPT testing,[43] the chance of misclassification between CBD or BeS and controls in our study was minimal because of the long latency from first exposure to the time of medical screening among our subjects, the lack of ongoing exposure because the facilities were closed, the longer duration of exposure in the controls despite matching on year of birth and because we routinely performed chest radiographs on all participants no matter what their BeLPT result, which were then evaluated by three B readers. All of the above factors would reduce the likelihood of controls being cases or becoming cases in the future, unlike studies in currently exposed cohorts where repeat screenings have identified additional exposed workers developing positive results.[44 45] One other issue related to the long latency from first exposure and time to medical screening in our cohort is that if there is an association between genetics and severity of CBD such that it affects survival, then those with the most progressive forms of CBD would had died prior to participation in our medical screening.

The results of this study continue to expand our knowledge about the genetics of beryllium disease. Specific HLA molecules definitively play a significant role in this immunologically mediated disease. Additional studies will need to focus on both the interaction of different HLA polymorphisms as well as the identification of other genetic factors besides HLA polymorphisms. Ideally these pursuits will contribute to interventions that will prevent the occurrence of beryllium disease as well as provide insight into other conditions with similar pathological mechanisms.

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Competing interests Dr Rossman has been an expert witness for beryllium related court cases.

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REFERENCES

1. **Newman LS,** Mroz MM, Balkissoon R, *et al*. Beryllium sensitization progresses to chronic beryllium disease: a longitudinal study of disease risk. *Am J Respir Crit Care Med* 2005;**171**:54–60.
2. **Sterner JH,** Eisenbud M. Epidemiology of beryllium intoxication. *Arch Ind Hyg Occup Med* 1951;**4**:123–51.
3. **Richeldi L,** Sorrentino R, Saltini C. HLA-DPB1 glutamate 69: a genetic marker of beryllium disease. *Science* 1993;**262**:242–4.
4. **Wang Z,** Farris GM, Newman LS, *et al*. Beryllium sensitivity is linked to HLA-DP genotype. *Toxicology* 2001;**165**:27–38.
5. **Rossman MD,** Stubbs J, Lee CW, *et al*. Human leukocyte antigen class II amino acid epitopes: susceptibility and progression markers for beryllium hypersensitivity. *Am J Respir Crit Care Med* 2002;**165**:788–94.
6. **Maier LA,** McGrath DS, Sato H, *et al*. Influence of MHC class II in susceptibility to beryllium sensitization and chronic beryllium disease. *J Immunol* 2003;**171**:6910–18.

7. **McCanlies EC,** Ensey JS, Schuler CR, *et al*. The association between HLA-DPB1Glu69 and chronic beryllium disease and beryllium sensitization. *Am J Ind Med* 2004;**46**:95—103.
8. **Richeldi L,** Kreiss K, Mroz MM, *et al*. Interaction of genetic and exposure factors in the prevalence of berylliosis. *Am J Ind Med* 1997;**32**:337—40.
9. **Saltini C,** Richeldi L, Losi M, *et al*. Major histocompatibility locus genetic markers of beryllium sensitization and disease. *Eur Respir J* 2001;**18**:677—84.
10. **Wang Z,** White PS, Petrovic M, *et al*. Differential susceptibilities to chronic beryllium disease contributed by different Glu69 HLA-DPB1 and - DPA1 alleles. *J Immunol* 1999;**163**:1647—53.
11. **Bill JR,** Mack DG, Falta MT, *et al*. Beryllium presentation to CD4+ T cells is dependent on a single amino acid residue of the MHC class II beta-chain. *J Immunol* 2005;**175**:7029—37.
12. **Fontenot AP,** Keizer TS, McCleskey M, *et al*. Recombinant HLA-DP2 binds beryllium and tolerizes beryllium—specific pathogenic $CD4^+$ T cells. *J Immunol* 2006;**177**:3874—83.
13. **Lombardi G,** Germain C, Uren J, *et al*. HLA-DP allele-specific T cell responses to beryllium account for DP-associated susceptibility to chronic beryllium disease. *J Immunol* 2001;**166**:3549—55.
14. **Amicosante M,** Sanarico N, Berretta F, *et al*. Beryllium binding to HLA-DP molecule carrying the marker of susceptibility to Berylliosis glutamate ß69. *Human Immunology* 2001;**62**:686—93.
15. **Amicosante M,** Berretta F, Dweik R, *et al*. Role of high-affinity HLA-DP specific CLIP-derived peptides in beryllium binding to the HLA-DPGlu69 berylliosis-associated molecules and presentation to beryllium-sensitized T cells. *Immunology* 2009; **128**(1 Suppl):e462—70.
16. **McCanlies EC,** Kreiss K, Andrew M, *et al*. HLA-DPB1 and chronic beryllium disease: a HuGE review. *Am J Epidemiol* 2003;**157**:388—98.
17. **Amicosante M,** Berretta F, Rossman M, *et al*. Identification of HLA-$DRPB^{Phe47}$ as the susceptibility marker of hypersensitivity to beryllium in individuals lacking the berylliosis-associated supratypic marker HLA-DPB^{E69}. *Respir Res* 2005;**6**:94.
18. **Sato H,** Spagnolo P, Silveira L, *et al*. BTNL2 allele associations with chronic beryllium disease in HLA-DPβ1*Glu 69- negative individuals. *Tissue Antigens* 2007;**70**:480—6.
19. **Jonth AC,** Silveira L, Fingerlin TE, *et al*. TGF-β1 variants in chronic beryllium disease and sarcoidosis. *J Immunol* 2007;**179**:4255—62.
20. **Gaede KI,** Amicosante M, Schurmann M, *et al*. Function associated transforming growth factor-ß gene polymorphism in chronic beryllium disease. *J Mol Med* 2005;**83**:397—405.
21. **Sato H,** Silveira L, Fingerlin T, *et al*. TNF polymorphism and bronchioalveolar lavage cell TNF-α levels in chronic beryllium disease and beryllium sensitization. *J Allergy Clin Immunol* 2007;**119**:687—96.
22. **McCanlies EC,** Schuler CR, Kreiss K, *et al*. TNF-α polymorphisms in chronic beryllium disease and beryllium sensitization. *J Occup Environ Med* 2007;**49**:446—52.
23. **Rosenman K,** Hertzberg V, Rice C, *et al*. Chronic beryllium disease and sensitization at a beryllium processing facility. *Environ Health Perspect* 2005;**113**:1366—72.
24. **Rosenman KD,** Monos D, Hertzberg VS, *et al*. Genetic susceptibility to beryllium toxicity. *Proceedings of the American Thoracic Society International Conference*. 18—23 May 2007, San Francisco, California. **175**:C18, A564.
25. **Dapprich J,** Magira E, Samonte MA, *et al*. Identification of a novel HLA-DPB1 allele (DPB1*1902) by haplotype-specific extraction and nucleotide sequencing. *Tissue Antigens* 2007;**69**:282—4.
26. **Bang H,** Zhou XK, Mazumdar M, *et al*. *Statistical Methods in Molecular Biology*. New York: Humana Press, Springer, 2010:540.
27. **Svejgaard A,** Ryder LP. HLA and disease associations: detecting the strongest association. *Tissue Antigens* 1994;**43**:18—27.
28. **Kanterakisa S,** Magiraa E, Rosenman KD, *et al*. SKDM human leukocyte antigen (HLA) tool: A comprehensive HLA and disease associations analysis software. *Hum Immunol* 2008;**69**:522—5.
29. **Snyder JA,** Demchuk E, McCanlies EC, *et al*. Impact of negatively charged patches on the surface of MHC class II antigen—presenting proteins on risk of chronic beryllium disease. *J R Soc Interface* 2008;**5**:749—58.
30. **Morel PA,** Dorman JS, Todd JA, *et al*. Aspartic acid at position 57 of the HLA-DQ beta chain protects against type I diabetes: a family study. *Proc Natl Acad Sci USA* 1988;**85**:8111—15.
31. **Magira EE,** Papaioakim M, Nachamkin I, *et al*. Differential distribution of HLA-DQ beta/DR beta epitopes in the two forms of Guillain-Barré syndrome, acute motor axonal neuropathy and acute inflammatory demyelinating polyneuropathy (AIDP): identification of DQ beta epitopes associated with susceptibility to and protection from AIDP. *J Immunol* 2003;**170**:3074—80.
32. **Fu XT,** Bono CP, Woulfe SL, *et al*. Pocket 4 of the HLA —DR (alpha, beta 1*0401) molecule is a major determinant of T cells recognition of peptide. *J Exp Med* 1995;**181**:915—26.
33. **Wucherpfennig KW,** Yu B, Bhol K, *et al*. Structural basis for major histocompatibility complex (MHC)-linked susceptibility to autoimmunity: charged residues of a single MHC binding pocket confer selective presentation of self-peptides in pemphigus vulgaris. *Proc Natl Acad Sci USA* 1995; **92**:11935—9.
34. **Zanelli E,** Breedveld FC, de Vries RR. HLA class II association with rheumatoid arthritis: facts and interpretations. *Hum Immunol* 2000;**61**:1254—61.
35. **Gregersen PK,** Silver J, Winchester RJ. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum* 1987;**30**:1205—13.
36. **Bielekova B,** Goodwin B, Richert N, *et al*. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83—99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med* 2000;**6**:1167—75.
37. **Greer JM,** Pender MP. The presence of glutamic acid at positions 71 or 74 in pocket 4 of the HLA-DRbeta1 chain is associated with the clinical course of multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2005;**76**:656—62.
38. **Smith KJ,** Pyrdol J, Gauthier L, *et al*. Crystal structure of HLA-DR2 (DRA*0101, DRB1*1501) complexed with a peptide from human myelin basic protein. *J Exp Med* 1998;**188**:1511—20.
39. **Zerva L,** Cizman B, Mehra NK, *et al*. Arginine at positions 13 or 70—71 in pocket 4 of HLA-DRB1 alleles is associated with susceptibility to tuberculoid leprosy. *J Exp Med* 1996;**183**:829—36.
40. **Monos DS,** Pappas J, Magira EE, *et al*. Identification of HLA-DQalpha and -DRbeta residues associated with susceptibility and protection to epithelial ovarian cancer. *Hum Immunol* 2005;**66**:554—62.
41. **Schuler CR,** Kent MS, Deubner DC, *et al*. Process-related risk of beryllium sensitization and disease in a copper-beryllium alloy facility. *Am J Ind Med* 2005;**47**:195—205.
42. **Tinkle SS,** Antonini JM, Rich BA, *et al*. Skin as a route of exposure and sensitization in chronic beryllium disease. *Environ Health Perspect* 2003;**111**:1202—8.
43. **Middleton DC,** Lewin MD, Kowalski PJ, *et al*. The BeLPT: algorithims and implications. *Am J Ind Med* 2006;**49**:36—44.
44. **Stange AW,** Furman FJ, Hilmas DE. The beryllium lymphocyte proliferation test: relevant issues in beryllium health surveillance. *Am J Ind Med* 2004;**46**:453—62.
45. **Newman LS,** Mroz MM, Maier LA, *et al*. Efficacy of serial medical surveillance for chronic beryllium disease in a beryllium machining plant. *J Occup Environ Med* 2001;**43**:231—7.

HLA class II DPB1 and DRB1 polymorphisms associated with genetic susceptibility to beryllium toxicity

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