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HLA-DPB POLYMORPHISMS: Glu 69 ASSOCIATION WITH SARCOIDOSIS

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SUMMARY

Sarcoidosis is a chronic granulomatous disorder, which is characterized by the accumulation of activated $CD4^{+}$ T lymphocytes (T cells) at disease sites. There is up-regulation of cell surface expression of MHC molecules in sarcoidosis, and it has been suggested that specific MHC class II alleles are associated with the disease. A study of chronic beryllium disease (CBD), a granulomatous disorder which is pathologically similar to sarcoidosis, has identified an association between this disease and the presence of a glutamine residue at position 69 (Glu 69+) of the B1 chain of the HLA-DPB molecule. A further study also suggested the importance of Glu at position 55 of the same chain. The aims of the present study were to attempt to define MHC class II alleles associated with sarcoidosis by comparison of their frequency in two groups of subjects and to compare the frequency of HLA-DPB1 Glu 69+/– and Glu 55+/– alleles in the same subjects. Forty-one subjects with sarcoidosis and 76 normal subjects were studied. The polymorphic regions of the class II MHC were identified by PCR in association with sequence-specific oligonucleotide probes. There were no significant differences in the phenotype frequencies of MHC class II or Glu 55+ alleles between the two groups of subjects. However, there was a significant increase ($P = 0.02$) in the frequency of HLA-DPB1* Glu 69+ alleles compared with the control population. We therefore suggest that the presence of a Glu residue at position 69 on the DPB1 chain may play an important role in antigen presentation and recognition in chronic granulomatous diseases.

INTRODUCTION

Sarcoidosis is a chronic inflammatory granulomatous disease of undetermined aetiology, affecting the lung in the majority of cases. The T lymphocyte (T cell) has been shown to play a critical role in the pathogenesis of the disease (Crystal *et al.*, 1981) and T-cell activation is restricted to the affected organs (Müller-Quernheim *et al.*, 1992). The presence of lymphocytic alveolitis, associated with an interstitial mononuclear cell infiltration of $CD4^{+}$ T cells at the sites of disease, is an early event in the

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sarcoid lung and is associated with a relative depletion of these cells in the peripheral blood (Crystal *et al.*, 1981; Mitchell & Scadding, 1974; Hunninghake & Crystal, 1981). $CD4^+$ T cells obtained by bronchoalveolar lavage (BAL) from the lower respiratory tract of subjects with sarcoidosis express cell surface activation markers, undergo spontaneous proliferation *in vitro* and spontaneously release cytokines (Konishi *et al.*, 1988; Robinson *et al.*, 1985; Saltini *et al.*, 1986; Pinkston *et al.*, 1983). This profile of activity may cause further $CD4^+$ T-cell proliferation and recruitment, monocyte influx from the local circulation and differentiation to macrophages, macrophage activation and granuloma formation. In human granulomatous diseases other than sarcoidosis and in animal models of granuloma formation, the immune response is believed to be the result of locally persistent poorly degraded antigen. By analogy, it has been suggested that, in sarcoidosis, persistent local stimuli at the disease site result in local $CD4^+$ T-cell activation via antigen-driven mechanisms leading no further $CD4^+$ recruitment, macrophage accumulation and activation followed by granuloma formation.

Chronic beryllium disease (CBD) is a granulomatous disease which develops in the lower respiratory tract in response to persistent exposure to beryllium. It occurs in less than 5% of the exposed population and is characterized by the accumulation of Be-specific $CD4^+$ T cells in the lungs which proliferate *in vitro* in the presence of beryllium salts (Rossman *et al.*, 1989). Clinically, it is often indistinguishable from sarcoidosis and it shares the accumulation of activated T cells at disease sites in response to persistent beryllium exposure.

The phenotype of the responsive T cell in an immune reaction correlates with the presentation of antigen in association with the cell surface major histocompatibility complex (MHC) molecule. $CD4^+$ T cells are responsive to antigens presented by class II MHC molecules, and recognition of the antigen/MHC complex by the T-cell receptor results in T-cell activation and proliferation which is characteristic of sarcoidosis and CBD.

In a study of the association between CBD and the MHC (Richeldi *et al.*, 1993), it was shown that the presence of a glutamine residue (Glu) at position 69 of the HLA-DPB1 chain is a marker of disease susceptibility. Additionally, another study showed that a Glu residue at position 55 of the HLA-DPB1 chain occurred only in beryllium-sensitive subjects (Stubbs *et al.*, 1994). These data clearly suggest a significant role for the HLA-DPB1 chain in susceptibility to berylliosis and, by association, with other chronic granulomatous diseases such as sarcoidosis.

The aim of this study was to investigate the association between HLA-DPB Glu 69+, Glu 55+ and also HLA-DR, -DQ and -DPB alleles and sarcoidosis in a clinically well-defined group of subjects.

MATERIALS AND METHODS

Subjects

Forty-one UK Caucasoid subjects with sarcoidosis were studied. The diagnosis of sarcoidosis was confirmed by typical histopathological findings from lung biopsy or by Kveim test and all subjects showed appropriate clinical features of sarcoidosis.

Control subjects

Seventy-six UK Caucasoid subjects without any history of sarcoidosis or other lung diseases were studied as controls.

Genomic DNA preparation

Genomic DNA was extracted from peripheral venous blood using a high-salt extraction technique (Miller *et al.*, 1988) with minor modifications. Briefly, whole blood, collected into 5% Na_2EDTA

(BDH Chemicals, Poole, Dorset, UK) in PBS (pH 7.35) and stored at −20°C, was thawed and washed with TE buffer [10 mM Tris base (Sigma Ltd., Poole, Dorset, UK), 1 mM Na_2EDTA, pH 8.0]. The mixture was centrifuged and the red blood cells were lysed by incubation with a swelling buffer (10 mM Tris-HCl, pH 7.4, 20 mM NaCl and 5 mM $MgCl_2$) at 4°C for 20 min before centrifugation (800*g*, 15 min, room temperature). The resultant pellet was then incubated in red cell and nuclei lysis buffers as previously described (Miller *et al.*, 1988). Finally, the DNA was precipitated with 5 M NaCl (1/10th volume) and 2 volumes of absolute ethanol. The DNA was washed with 70% ethanol before resuspension in TE. The concentration of DNA was determined by measuring the ultraviolet absorption at 260 nm.

PCR-SSO analysis

The second exons of the HLA-DRB1, -DQA1*, -DQB1* and -DPB1* chains were amplified by PCR using the specific generic HLA primer pairs supplied by the British Society for Histocompatibility and Immunogenetics (BSHI).

Genomic DNA (1 µg) was mixed with PCR buffer (Boehringer Mannheim, Lewes, E. Sussex, UK), 0.2 mM each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxyadenosine triphosphate and deoxythymidine triphosphate (Pharmacia, St. Albans, Herts, UK), 1 µg of each primer and 3 U Taq DNA polymerase (Boehringer Mannheim) in a final volume of 50 µl. PCR amplification was automated with a thermal cycler (Techne, Cambridge, UK). The initial denaturation was for 3 min at 95°C, followed by 30 cycles of 95°C for 1.3 min, 55°C for 2 min and 72°C for 2 min. The final extension was maintained for 10 min. Ten percent of the reaction volume was then electrophoresed on a 1.2% agarose gel containing 0.1% w/v ethidium bromide (Sigma Ltd). The amplified DNA was denatured and transferred to Hybond-N+ nylon membranes (Amersham plc., Amersham, Buck, UK) by the Southern blotting procedure (Southern, 1975). The membranes were washed in saline sodium citrate solution (0.75 M NaCl and 0.1 M sodium citrate; BDH Chemicals) and cross-linked by UV irradiation, using an automatic cross linker (Hybaid, Teddington, Middlesex, UK).

The 5' biotinylated HLA-DRB1, -DQA1*, -DQB1* and -DPB1* sequence-specific oligonucleotide probes (SSOs) (BSHI) were each hybridized with the cross-linked membranes. The membranes were prehybridized for 30 min in hybridization buffer (as described by Amersham for ECL 3' oligolabelling and detection) at a temperature dependent on the length of the SSO (SSOs of 17 or 18 bases in length were hybridized at 48°C and SSOs of 21 bases were hybridized at 52°C). The oligonucleotide probe was added to the hybridization buffer (400 ng oligonucleotide/10 mL hybridization buffer) and incubated for 2 h in a shaking water bath. The membranes were washed for 20 min at the hybridization temperature in saline sodium citrate solution (3 M NaCl and 0.5 M sodium citrate) containing 0.5% SDS (Sigma Ltd) and then twice for 15 min each in 3 M tetramethylammonium chloride containing 0.1% SDS, 50 mM Tris, pH 8.0 and 2 mM EDTA at 54°C for SSOs of 17 and 18 bases in length and at 58°C for 21-base oligonucleotides. The membranes were blocked as described by the manufacturer (Amersham) prior to incubation with streptavidin-HRP conjugate (Amersham) for 30 min at room temperature. Signal generation and detection were carried out as described by the manufacturer, and autoradiography was performed by exposure to Kodak XAR-5 film (Sigma Ltd.).

Statistical analysis

The data were tested for significance by chi-square analysis with correction for the number of analyses performed where appropriate.

RESULTS

To confirm that any differences in the phenotype frequency of any HLA allele were related to the disease and not to a bias caused by selection of our control population, the HLA phenotype frequency of the control population described here was compared with that of previously published populations, for which the HLA phenotypes were determined using PCR-SSO analysis. HLA-DRB, -DQA and -DQB phenotype frequencies were compared with those published by Doherty *et al.* (1992) and HLA-DPB frequencies were compared with those published by Stephens *et al.* (1993). There were no significant differences in the phenotype frequencies of any HLA allele between our control population and those previously published.

There was a significant increase ($P = 0.02$, corrected for the number of comparisons made) in the phenotype frequency of Glu 69+ HLA-DPB1* phenotypes in the subjects with sarcoidosis compared with the control population. Twenty-six of the 41 subjects with sarcoidosis were HLA-DPB1* Glu 69+ compared to 29 of the 76 control subjects. There was no significant difference ($P > 0.05$) in the frequency of Glu 55+ alleles between the two groups. To establish whether the increase in Glu 69+ alleles was due to a preponderance of one particular HLA-DPB phenotype, as previously suggested (Richeldi *et al.*, 1993), we determined the phenotype frequencies of all the HLA-DPB alleles by PCR-SSO analysis. However, there were no significant differences between the two groups of subjects after correction for the multiple comparisons made (Table 1).

To establish whether the increase in the frequency of Glu 69+ alleles was related to a more complex or ancestral phenotype involving HLA-DRB, DQA and DQB, we also determined the frequencies of these alleles.

There were no significant differences in the frequencies of any HLA-DRB1* phenotype between the two groups of subjects (Table 2). However, following analysis of the frequency of HLA-DQA alleles, there was a significant difference in the frequencies of HLA-DQA1*0103 ($P = 0.005$), -0201 ($P = 0.04$), and -0501 ($P = 0.02$) between the two groups of subjects, as shown in Table 3. For this

TABLE 1. Comparison of HLA-DPB1 phenotype frequencies between subjects with sarcoidosis and control subjects

	Phenotype frequency (%)	
DPB1* phenotype	Sarcoidosis subjects	Control subjects
0101	9.76	9.2
*0201	19.51	9.2
*0202	14.63	10.5
0301	26.83	26.3
0401	53.66	65.8
0402	19.51	17.1
0501	2.44	15.8
*0601	7.31	6.6
*0801	2.44	2.6
*0901	2.44	0
*1001	12.19	7.9
1101	4.88	5.3
*1301	4.88	0
1401	7.31	7.9
1501	0	7.9
1801	4.88	1.3
*1901	0	0

*Glu 69+ alleles.

TABLE 2. Comparison of HLA-DRB1 phenotype frequencies between subjects with sarcoidosis and control subjects

	Phenotype frequency (%)	
DRB1* phenotype	Sarcoidosis subjects	Control subjects
0101-3	17.07	25.0
1501, 1502, 1601, 1602	46.34	32.89
0301, 0302	24.39	25.0
0401-0411	31.71	42.11
1101-4, 1201, 1202	28.13	14.47
1301-5, 1401-5	34.15	32.37
0701	12.20	25
0801-4	5.13	5.26
0901	0	2.63
1001	0	1.32

locus, nine analyses were performed and consequently only a significance value of less than 0.005 was acceptable for rejection of the null hypothesis, and therefore these differences were rejected as non-significant. Similarly, before correction for the number of analyses performed, there was a significant difference in the phenotype frequencies of HLA-DQB1*0401 and HLA-DQB1*0201 (Table 4) between the sarcoidosis group and the control population ($P=0.02$ and $P=0.04$, respectively). For this locus, 15 analyses were performed, and the null hypothesis could only be rejected if the significance value was less than 0.003. Therefore, after correction for the number of analyses performed, these differences failed to reach statistical significance.

DISCUSSION

We have shown a significant increase in the phenotype frequency of Glu 69+ HLA-DPB1 alleles in subjects with sarcoidosis when compared with control subjects. These data parallel the previously reported study of CBD (Richeldi *et al.*, 1993), in which the presence of a Glu amino acid residue at position 69 was associated with susceptibility to CBD. However, we did not find an association between HLA-DPB1 Glu 55+ alleles and sarcoidosis as reported by other workers (Stubbs *et al.*, 1994). The association of the Glu 69 and Glu 55 amino acid residues with CBD may indicate the importance of

TABLE 3. Comparison of HLA-DQA1 phenotype frequencies between subjects with sarcoidosis and control subjects

	Phenotype frequency (%)	
DQA1* phenotype	Sarcoidosis subjects	Control subjects
0101	14.63	30.26
0102	41.46	36.84
0103	24.39	9.21
0201	9.76	23.68
0301	2.44	5.26
0401	58.54	40.79
0501	4.88	1.32
0601	31.71	43.42

TABLE 4. Comparison of HLA-DQB1 phenotype frequencies between subjects with sarcoidosis and control subjects

	Phenotype frequency (%)	
DQB1* phenotype	Sarcoidosis subjects	Control subjects
0501	15.38	26.32
0502	5.13	2.63
0503	0	5.26
0504	5.13	2.63
0601	7.69	2.63
0602	35.90	28.95
0603	12.82	6.58
0604	10.26	5.26
0201	25.64	40.79
0301	38.46	34.21
0302	15.39	28.95
0303	2.56	1.32
0401	7.69	1.32
0402	10.26	5.26

these residues in the orientation of a beryllium hapten in the binding cleft of the HLA-class II molecule. However, the data presented in our study suggest that HLA-DPB1 Glu 69+ alleles may also be important in other granulomatous disorders. The amino acid residue at position 69 may be important in the presentation of an antigen to the T-cell receptor, resulting in an immune response and possibly fibrosis, or may be acting as a marker in close linkage with other genes within the MHC region. To explore these hypotheses it would be necessary to perform larger, more relevant multi-centre studies and to study the HLA-DPB phenotype frequencies in other diseases involving granulomatous lesions such as tuberculosis.

Despite the significant increase in the frequency of HLA-DPB1 Glu 69+ alleles, there was no absolute association between any HLA phenotype and sarcoidosis in our study, although there are several possible reasons for this. It is perhaps relevant to recall that, in contrast to CBD, the causal agent in sarcoidosis has yet to be identified and it is therefore possible that there may be one or more antigens involved in development of the disease or that the antigen is complex in nature and may be presented in association with a number of HLA molecules to $CD4^+$ T cells. Additionally, we performed a large number of analyses in this study and were therefore constrained by the nature of statistical analysis to reject as non-significant several differences between the two groups of subjects. Although these differences just failed to reach significance after correction for the analyses undertaken, it may prove interesting to analyse another group of subjects with sarcoidosis prospectively for these alleles to establish the biological importance of these differences either individually or as a result of the strong linkage disequilibrium which is present within the MHC.

Data presented in previous studies of HLA associations with sarcoidosis have shown a marked lack of concordance of data (Ina *et al.*, 1989; Abe *et al.*, 1987; Lenhart *et al.*, 1990; Ikeda *et al.*, 1992; Grunewald *et al.*, 1992; Ishihara *et al.*, 1994; Kunikane *et al.*, 1994). In this study we have investigated only subjects in whom sarcoidosis was confirmed by histopathological results and by clinical examination. Additionally, all of our subjects and controls were UK Caucasoid subjects. We have therefore minimized the confounding factors of inappropriate diagnosis and diverse ethnic origin.

We conclude from this study that the HLA-DPB locus is potentially of importance in sarcoidosis as well as CBD and possibly other granulomatous diseases, either alone or by association with other genes within the MHC complex. Further carefully designed studies are needed to confirm the association between Glu 69+ alleles and sarcoidosis and to explore the relationship with chronic progressive sarcoidosis.

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