The Journal of Immunology

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J Immunol 2001; 166:3549-3555; ;
doi: 10.4049/jimmunol.166.5.3549
http://www.jimmunol.org/content/166/5/3549

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

HLA-DP Allele-Specific T Cell Responses to Beryllium Account for DP-Associated Susceptibility to Chronic Beryllium Disease[1]

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Occupational exposure to small molecules, such as metals, is frequently associated with hypersensitivity reactions. Chronic beryllium (Be) disease (CBD) is a multisystem granulomatous disease that primarily affects the lung, and occurs in ~3% of individuals exposed to this element. Immunogenetic studies have demonstrated a strong association between CBD and possession of alleles of HLA-DP containing glutamic acid (Glu) at position 69 in the HLA-DPβ-chain. T cell clones were raised from three patients with CBD in whom exposure occurred 10 and 30 years previously. Of 25 Be-specific clones that were obtained, all were restricted by HLA-DP alleles with Glu at DPβ69. Furthermore, the proliferative responses of the clones were absolutely dependent upon DPβ Glu^{69} in that a single amino acid substitution at this position abolished the response. As befits a disease whose pathogenesis involves a delayed type hypersensitivity response, the large majority of Be-specific clones secreted IFN-γ (Th1) and little or no IL-4 (Th2) cytokines. This study provides insights into the molecular basis of DP2-associated susceptibility to CBD. ***The Journal of Immunology*****, 2001, 166: 3549–3555.**

Occupational exposure to metals is associated with a variety of hypersensitivity reactions (1). In particular, contact with beryllium (Be) can lead to a chronic granulomatous lung reaction (2, 3). Chronic beryllium disease (CBD)[3] affects 1–5% of exposed workers and may affect bystanders (4). However, disease prevalence has often been underestimated because of the long latency period of the disease (months to decades) and its elusive clinical and pathological presentation (5). Some aspects of this pathology are similar to the syndrome named "hard metal disease" caused by the inhalation of metal dust containing cobalt (Co) (6).

In 1970 Hafin et al. showed that mononuclear cells from CBD patients react against Be in vitro (7). In 1990 we demonstrated the involvement of CD4$^+$ T cells in CBD by showing an accumulation of Be-specific CD4$^+$ T cells in the lung of CBD patients (8, 9). The specificity of these CD4$^+$ T cells was further confirmed in vitro by showing their reactivity to Be and the lack of responses to other metals (8). Similar results were obtained in different contexts with nickel (Ni) and gold (Au) by showing T cell reactivity to these metals from PBMC of patients with contact sensitivity reactions (10, 11).

It has long been known that allelic polymorphism in MHC genes determines immune response variation to individual Ags including autoantigens (12). Recently, we have investigated the involvement of MHC class II genes in the susceptibility to Be disease. Analysis of MHC class II genes in patients with CBD has shown a positive association with *HLA-DPB1*0201* and a negative association with *HLA-DPB1*0401* (13). Furthermore, sequence comparison of DPβ-chains suggested that susceptibility to CBD was conferred by expression of DPβ alleles with glutamic acid (Glu) at position 69. A subsequent population study demonstrated that the carriage of Glu^{69} was associated with an almost 10-fold increase of disease risk in exposed individuals (14). In this regard, *HLA-DPB1*0201* is the most common allele with Glu at this position; however, 15 of 32 HLA-DP alleles carry this polymorphism. Interestingly, we have recently shown an identical HLA-DP association in patients with hard metal disease. All patients in that study possessed a DPβ-chain with Glu at position 69 as compared with 17 of 35 exposed unaffected individuals (15).

In this study, we have investigated the functional basis of HLA-DP susceptibility in Be disease by generating T cell clones from patients with CBD. We found that all the Be-specific T cell clones obtained were restricted by HLA-DP alleles with Glu at DPβ69, and that a single amino acid substitution at residue 69 in the β-chain of the HLA-DP molecule completely abrogated their responses. These data elucidate the molecular basis of DP-associated susceptibility to CBD.

Materials and Methods

Patients

Three male patients named, respectively, TDC, FC, and NG, with CBD were identified through the University of Wales College of Medicine and the Royal Brompton Hospital (London, U.K.). The HLA-DR, -DQ, and -DP typing for the three patients is indicated in Table I.

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Received for publication August 28, 2000. Accepted for publication November 28, 2000.

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[1] This work was in part supported by the European Economic Community Biomed 1 Contract BMHI-CT92-0934, European Economic Community Environment Contract EVSV-CT92-0208, and U.S. Department of Energy Contract DE-FG02-93ER61714. C.G. was supported by a Medical Research Council Program Grant.

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[3] Abbreviations used in this paper: CBD, chronic beryllium disease; BAL, bronchoalveolar lavage; B-LCL, B lymphoblastoid cell line; Xth IHW, Xth International Histocompatibility Workshop; PI, propidium iodide; CD, celiac disease.

0022-1767/01/$02.00

Table I. *PBMC from CBD patients proliferated to Be*[a]

Patient Name	MHC Class II Tissue Typing	Proliferation to Be	
		− Be	+ Be
TDC	DRB1*1501, 1101; DQB1*06–, 07–; DPB1*0201	457 ± 181	134,283 ± 27,599
FC	DRB1*0301, 0701; DQB1*0201; DPB1*0201, 1101	322 ± 67	3,447 ± 617
NG	DRB1*0301, 04–; DQB1*0201, 0302; DPB1*0201, 0601	253 ± 21	34,701 ± 2,632

[a] PBMC derived from CBD patients typed for HLA class II molecules as indicated were cultured for 5 days with Be. Proliferative responses in the presence and in the absence of Be are shown.

Be and mitogen

The beryllium sulfate tetrahydrate ($BeSO_4 \cdot 4H_2O$), cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), and nickel (II) sulfate 7-hydrate ($NiSO_4 \cdot 7H_2O$) were purchased from Sigma-Aldrich (Dorset, U.K.).

Monoclonal Abs

The B7/21 (anti-HLA-DP; American Type Culture Collection (ATCC), Manassas, VA), L2 (anti-HLA-DQ, ATCC), and L243 (anti-HLA-DR, ATCC) mAbs were used after purification on protein A-Sepharose beads by standard methods. Eluted Ab was dialyzed against three changes of PBS.

Cell lines

EBV-transformed B lymphoblastoid cell lines (B-LCLs) were obtained from the Xth International Histocompatibility Workshop (Xth IHW). HLA-$DP2^+$ B-LCLs (9045: DRB1*1104/1201, DPA1*01, DPB1*02012/0402; 9038: DRB1*1201, DPA1*01, DPB1*02012; 9039: DRB1*1102, DPA1*01, DPB1*02012; 9036: DRB1*1101, DPA1*01, DPB1*02012) and HLA-$DP2^-$ B-LCLs (9037: DRB1*1101, DPA1*-, DPB1*0402; 9043: DRB1*1101, DPA1*0201, DPB1*1001; 9001: DRB1*0101, DPA1*-, DPB1*0402; 9013: DRB1*1501, DPA1*01, DPB1*0402) were used for this study. B-LCLs were cultured in RPMI 1640 tissue culture medium (Life Technologies, Paisley, U.K.) supplemented with 10% FCS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin in 25-cm^2 flasks, and were regularly passaged.

Murine DAP.3 cell transfectants expressing either HLA-DPB1*0201 (workshop number 8301) or HLA-DPB1*0402 (workshop number 8305) were obtained from the Xth IHW. They were maintained in DMEM supplemented with 10% FCS, 0.2% sodium bicarbonate, 2 μM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and MXH (6 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine) to maintain expression of the transfected genes. Cells were grown in 25-cm^2 flasks and passaged, following trypsinization, twice weekly.

Generation and maintenance of Be-specific T cell clones

PBMC were isolated from heparinized whole blood from the three patients by density centrifugation on a Lymphoprep gradient (Nycomed, Birmingham, U.K.). PBMC were cultured with $BeSO_4 \cdot 4H_2O$ in 24-well plates (Costar, High Wycombe, U.K.). After 6 days, the cultures were enriched for lymphoblasts by centrifugation on Ficoll-Hypaque and cultured for two more weeks in the presence of Be-pulsed autologous irradiated PBMC and 20 U/ml rIL-2 (Boehringer Mannheim, East Sussex, U.K.). The T cells were then cloned by limiting dilution at 0.3–1 cell/well in Terasaki trays (Greiner, Gloucester shine, U.K.) in the presence of PHA (2 μg/ml) and 10^4 allogeneic irradiated PBMC and rIL-2. After 10 days, cell growth was detected microscopically, and the contents of wells with growing cells were expanded further in medium with PHA, allogeneic PBMC, and rIL-2. The clones were maintained in culture by weekly stimulation with PHA, allogeneic PBMC, and rIL-2, in RPMI 1640 medium supplemented with 10% human serum, 2 μM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. T cell clones were used for functional assays between 1 and 2 wk after their last stimulation.

Proliferation assay

PBMC (10^5 cells/well) were cultured with different doses of Be. T cell clones (10^4 cells/well) were cultured with either B-LCL (3×10^4 cells/well) or DP-expressing murine DAP.3 transfectants (3×10^4 cells/well) pulsed with $BeSO_4 \cdot 4H_2O$ for 4 h and then treated with 120 Gy X-irradiation or with mitomycin C (50 μg/ml), respectively. The cells were plated out in flat-bottom microtiter plates, in a total volume of 200 μl, in RPMI 1640 medium supplemented with 10% human serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Wells were pulsed with 1 μCi of [^{3}H]TdR (Amersham International, Amersham, U.K.), after either 5 days (PBMC) or 48 h (T cell clones), and the cultures were harvested onto glass fiber filters 18 h later. Proliferation was measured as [^{3}H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures.

Annexin V staining

The B-LCL 9036 was pulsed with $BeSO_4.4H_2O$ for 4 h and irradiated. The B-LCL were then incubated either in the absence or in the presence of anti-HLA-DP mAb (B7/21) or anti-HLA-DR mAb (L243) at a concentration of 10 μg/ml. The cells were then stained at various time points (45 min, and 1.5 and 3 h) with annexin V-FITC and the vital dye propidium iodide (PI) to assess apoptosis. The cells were then analyzed by flow cytometry on a Becton Dickinson (Mountain View, CA) FACSCalibur.

Lymphokine production

Supernatants were collected after 24 h of T cell culture. Th1 and Th2 cytokines were measured using a standard ELISA. IFN-γ primary and secondary Abs were purchased from AMS Biotechnology (Oxon, U.K.), and IL-4 Abs were obtained from BioSource (Hertfordshine, U.K.). Briefly, primary Abs were coated overnight onto 96-well plates. The plate was then washed, and 1% BSA (Sigma-Aldrich) was used as the blocking reagent for 2 h. After washing, samples for testing were added to the wells along

Table II. *Proliferative responses to Be of T cell clones derived from CBD patients*[a]

T Cell Clones	Proliferative Responses (Δ cpm)	
	− Be	+ Be
TDC1	0	8,941
TDC3	0	28,467
TDC4	0	13,091
TDC5	0	62,427
TDC6	0	8,290
TDC13	0	32,499
TDC14	136	8,083
TDC17	740	10,040
TDC22	817	15,974
TDC30	0	9,551
TDC38	231	10,986
Be7	22	8,598
Be13	464	19,788
Be23	1,370	48,217
Be24	1,223	29,773
Be31	1,666	5,368
Be35	847	6,178
Be36	0	28,567
Be41	223	15,146
Be73	143	5,826
P1.1	0	6,687
P1.2	0	32,131
P1.15	67	4,020
P2.2	108	23,704
P2.8	8	31,194

[a] T cell clones derived from patient TDC, FC (Be and P1), and NG (P2) were cultured for 3 days with B-LCL 9036 prepulsed with Be. Proliferative responses in the absence and presence of Be are shown.

with biotinylated secondary Ab. After 2 h, wells were washed and streptavidin peroxidase conjugate (BioSource) was added for 45 min. Wells were washed thoroughly, and tetramethylbenzidine substrate was added. This led to color development, which was stopped using H_2SO_4. The plates were then read at 540 nm.

RNA extraction and cDNA synthesis

Cells (5×10^5) were placed in a sterile Eppendorf tube for RNA extraction. RNA was extracted using the method of Chomczynski and Sacchi (16). To each tube, 1 ml of cold RNAzol was added and the tube was vortexed. After a 5-min incubation on ice, 100 μl of chloroform was added, and the samples were centrifuged for 15 min at 4°C. The upper phase was then transferred to a clean tube containing an equal volume of isopropanol and 10 μg of transfer RNA as a carrier. After a 15-min incubation on ice, the sample was again centrifuged at 4°C for 15 min, the supernatant was removed, and the RNA pellet was washed with 1 ml of cold ethanol. The air-dried pellet was resuspended in 10 μl of autoclaved distilled water and stored at −70°C. cDNA was synthesized using oligo-dT-primed, Moloney murine leukemia virus-derived reverse transcriptase (Life Technologies) and 5 μl of RNA sample in 20-μl reactions. cDNA reactions were diluted to 100 μl and stored at −70°C; 2 μl of diluted cDNA was used for each PCR.

PCR amplification and sequencing

For analysis of TCR V-gene usage cDNAs were amplified using a panel of primers specific for TCR Vα or Vβ families in combination with a Cα or Cβ primer. TCR primer sequences were as in Reference (17). Samples were amplified by PCR for 30 cycles (30 s at 94°C, 30 s at 64°C, and 30 s at 72°C) for both TCR Vβ and TCR Vα primers. The TCR Cβ reverse primer sequence was: 5′-GGCAGACAGGACCCCT TGCTGGTAGGACAC-3′. The TCR Cα reverse primer sequence was: 5′-ACTTTGTGACACATTTGTTTGAG-3′.

The amplified fragments were separated by agarose gel electrophoresis and stained with ethidium bromide. The specific bands were cut, and the amplified products were purified using a gel band purification kit (Pharmacia Biotech, Uppsala, Sweden). Direct sequencing was performed using a primer upstream of the TCR Cβ reverse primer: 5′-TGTGCACCTCCTTCCCATTCA-3′ and a primer upstream of the TCR Cα reverse primer: 5′-AGGCAGACAGACTT GTCACTG-3′. A PCR-based sequence kit (Perkin-Elmer, Norwalk, CT) was used following the manufacturer's instructions and using [^{32}P]dATP as isotope; 1 μl of each sequencing reaction was loaded onto a 6% urea-polyacrylamide gel, and the bands were separated by electrophoresis. The gels were dried and analyzed by autoradiography.

Results

T cell clones generated from patients with CBD are specific for Be

Three patients with CBD were selected for this study. They all expressed the susceptibility-conferring Glu at position 69 in one or both of their DPβ-chains. PBMC were separated and cultured with different doses of Be. Dose-dependent T cell proliferation was seen in all three patients. In Table I is shown the proliferation of PBMC to the optimal dose of Be. PBMC from healthy controls did not show any significant response to Be, although three of six control individuals carried HLA-DP alleles with glutamic acid at position 69 in the DPβ-chain (data not shown).

T cell lines and clones were generated from CBD patients by culturing PBMC with $BeSO_4 \cdot 4H_2O$, as described in *Materials and Methods*. The T cell clones obtained were CD4 positive and, first, they were tested against $BeSO_4 \cdot 4H_2O$. Of the T cell clones obtained, 65% (11/17) from patient TDC, 35% (13/37) from patient FC, and 30% (3/10) from patient NG were specific for Be. Their reactivity to different doses of Be was then investigated using homozygous B-LCLs from the Xth IHW as APCs. In Table II is shown the summary of the proliferative responses to the optimal concentration of Be of 11 T cell clones from patient TDC, 12 T cell clones from patient FC, and two clones from patient NG. The dose response of four T cell clones, two from TDC and two from FC, are shown in Fig. 1, *a–d*.

To address whether presentation of any other metals, such as Co, could lead to the stimulation of Be-specific T cells, T cell clones were cultured with B-LCL pulsed with either Co or Ni. T cell clones proliferated only in the presence of Be (Fig. 1, *e* and *f*).

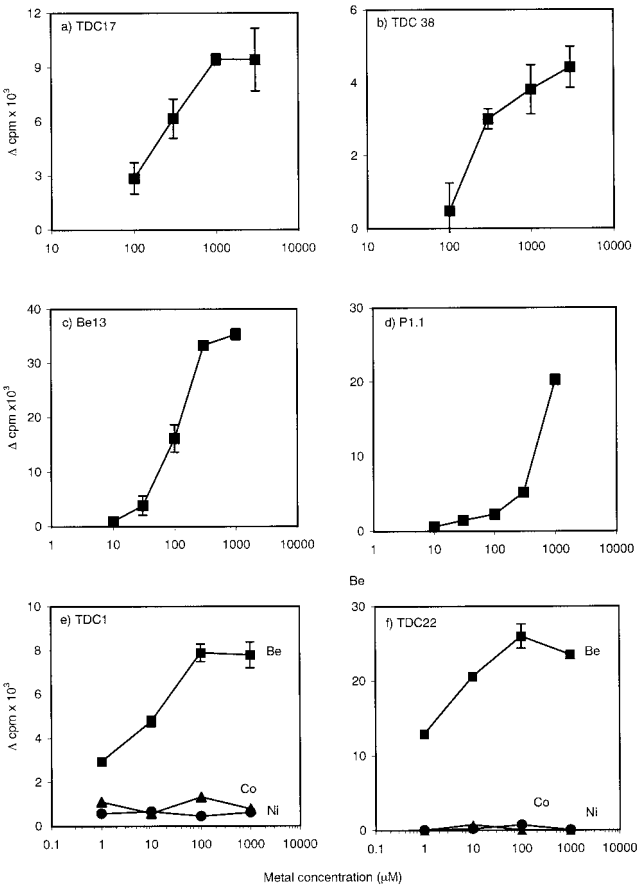

FIGURE 1. T cell clones generated from patients with CBD respond specifically to Be in a dose-dependent manner. T cell clones, TDC17 (*a*), TDC38 (*b*), Be 13 (*c*), and P1.1 (*d*), were cultured (10^4 cells/well) with HLA-DP2-expressing B-LCL, 9036 (3×10^4 cells/well) prepulsed with different doses of $BeSO_4.4H_2O$. *e* and *f*, T cell clones, TDC1 and TDC22, were cultured with B-LCL 9036 prepulsed with Be (■), Co (▲), and Ni (●). After 48 h [^{3}H]TdR was added and the plates were harvested 18 h later. Proliferation is expressed as Δcpm.

T cell clones specific for Be are restricted by HLA-DP

The restriction element used by the Be-specific T cell clones was first examined by using a panel of homozygous B-LCLs from the Xth IHW as APCs. In Fig. 2 is shown the proliferation of three representative Be-specific T cell clones. All the T cell clones tested responded to Be only when it was presented by B-LCLs expressing HLA-DPB1*0201. No response was detected to DPB1*0201-negative B-LCLs expressing either HLA-DRB1*1101 (patient TDC) or HLA-DRB1*0301 (patient FC) (Fig. 2 and data not shown).

The involvement of HLA-DP molecules in the responses of T cell clones derived from the above lines to Be was further investigated using mAbs specific for HLA-DR, -DQ, and -DP. The results for four representative Be-specific T cell clones are shown in Fig. 3, *a–c*. All the T cell clones tested were completely inhibited by the presence of anti-HLA-DP mAb. In contrast, no inhibition was observed with the mAb against HLA-DQ molecules. In all the experiments performed, the presence of the anti-HLA-DR mAb (L243) consistently led to 30–50% inhibition. First, to exclude the possibility that the inhibition with the anti-DR mAb reflected nonclonality in the Be-specific T cell populations, T cell clones were further subcloned by limiting dilution. No differences were observed in the degree of anti-HLA-DR inhibition of the subclones when compared with the original T cell population (data not shown). The alternative hypothesis tested was that the inhibition

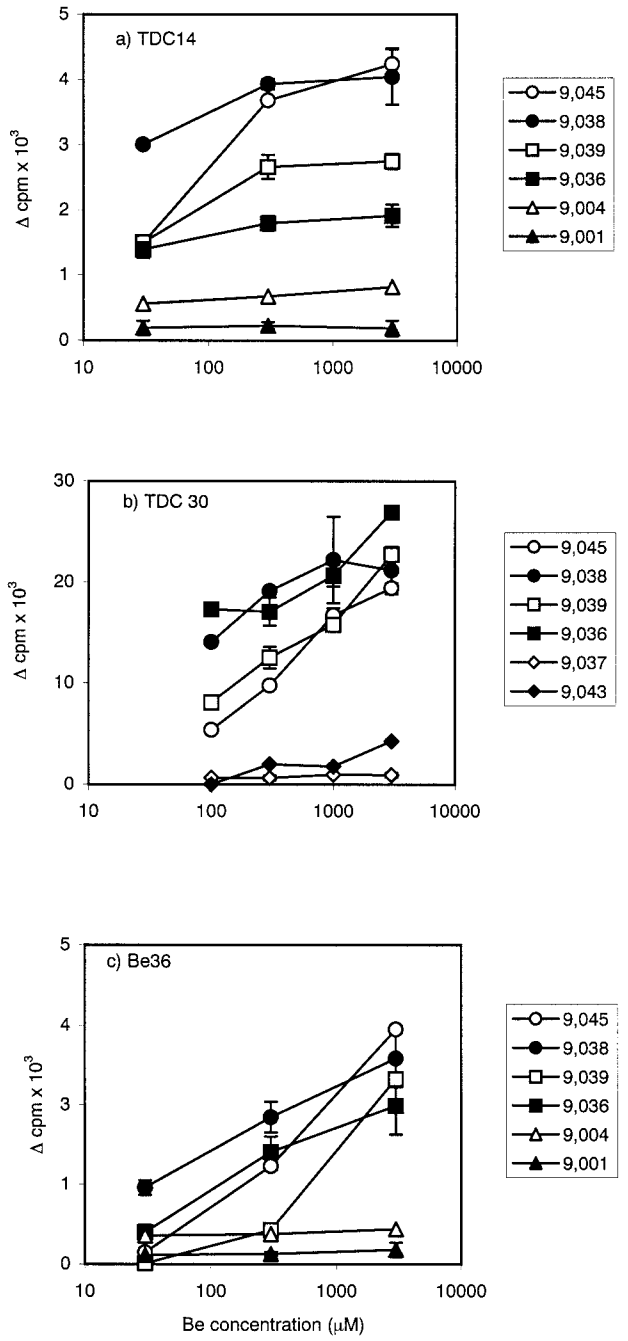

FIGURE 2. Be-specific T cell clones are HLA-DP restricted. T cell clones, TDC14 (*a*), TDC30 (*b*), and Be36 (*c*) were cultured (10^4 cells/well) with a panel of B-LCLs (3×10^4 cells/well) prepulsed with different doses of $BeSO_4 \cdot 4H_2O$. HLA-$DP2^+$ B-LCLs from the Xth IHW (9045, 9038, 9039, 9036) and HLA-$DP2^-$ B-LCLs from the Xth IHW (9037, 9043, 9001) were used. Proliferation is expressed as Δcpm.

observed with the anti-DR mAb was due to cell death of the B-LCL presenting Be. This hypothesis is in agreement with the work of Drenou et al. (18). They showed, in a slightly different system, that cross-linking of MHC-class II molecules on a B cell lymphoma led to apoptosis (18). To address this possibility, B-LCL were incubated with the anti-DR mAb (L243) for different lengths of time as described in *Materials and Methods*, and the percentage of apoptotic and necrotic cells was measured using PI and annexin V staining. After 45 min nearly 10% of the cells were undergoing apoptosis and close to 20% had already died (data not shown).

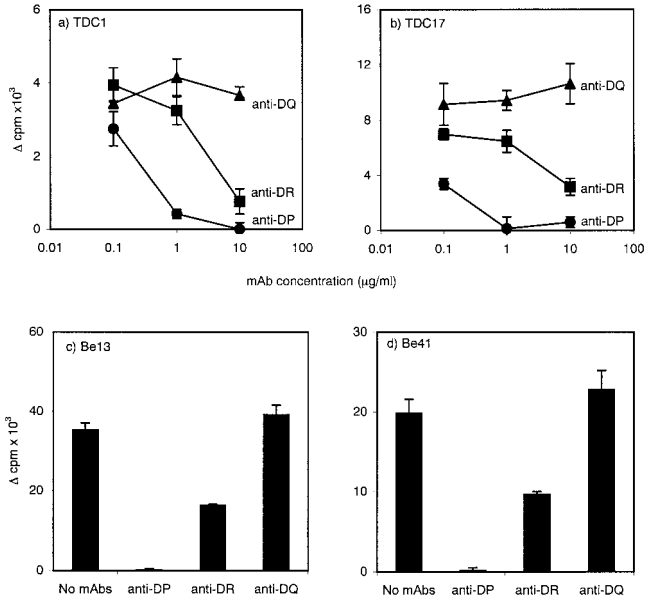

FIGURE 3. T cell clones specific for Be are inhibited by anti-DP mAb, but also by anti-HLA-DR mAb. T cell clones specific for Be (10^4 cells/well), TDC17 (*a*), TDC30 (*b*), Be 13 (*c*), and Be 41 (*d*) were cultured with 3×10^4 cells/well of Be-prepulsed B-LCL in the presence of anti-HLA class II mAbs anti-DQ, anti-DR, and anti-DP, as indicated. Proliferation is expressed as Δcpm.

After 3 h the addition of anti-DR (L243) mAb induced >30% cell death and another 14% undergoing apoptosis (Fig. 4, *e* and *f*). In contrast, anti-DP mAb (Fig. 4, *c* and *d*), which inhibited T cell proliferation, did not cause any cell death compared with untreated cells (Fig. 4, *a* and *b*).

Position 69 in the DPβ-chain is critically important in T cell recognition of Be

The genetic analysis of patients with CBD has revealed an association with alleles of HLA-DPB1 encoding a DPβ-chain with glutamic acid at residue 69. To investigate the functional relevance of Glu at residue 69 in the β-chain, we tested the response of Be-specific T cell clones to B-LCLs expressing either HLA-DPB1*0201 or HLA-DPB1*0402 that shared the same DPα-chain and differ only at position 69 in the β-chain. All the Be-specific T cell clones obtained responded only to the B-LCL with Glu at residue 69. The dose-dependent proliferation for four representative T cell clones is shown in Fig. 5, *a–d*.

The MHC restriction of the Be-specific T cell clones and, in particular, the importance of Glu at residue 69 were further investigated using murine DAP.3 cells transfected with cDNA clones encoding either HLA-DPB1*0201 (8301) or HLA-DPB1*0402 (8305). Only 3 of 12 T cell clones tested were capable of responding to the DAP.3-DP2 transfectants. In Fig. 5 are shown the proliferative responses of Be 23 (*e*) and Be41 (*f*) to different doses of Be presented by DAP.3-DP2 transfectants. No proliferation was seen to DAP.3 cells expressing comparable levels of DPB1*0402 (Fig. 5). Altogether, these data suggest that HLA-DP is the restriction element for Be-specific T cells and that the presence of Glu at residue 69 is essential for the reactivity of these T cell clones to Be.

The Be-specific T cell clones are predominantly Th1 cells with biased TCR usage

It has been shown previously that delayed type hypersensitivity reactions are mediated by T cells of the Th1 phenotype. In this

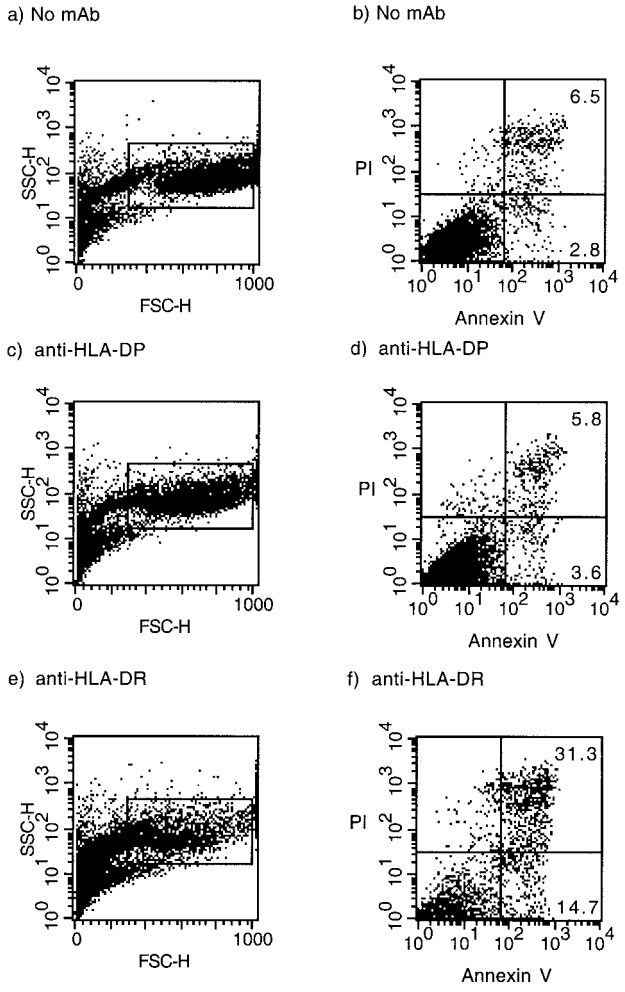

FIGURE 4. Anti-HLA-DR mAb induces cell death of the APC. B-LCL 9036, pulsed with Be and irradiated, were then incubated for 3 h either in the absence (*a* and *b*) or in the presence of anti-HLA-DP mAb (B7/21) (*c* and *d*) or anti-HLA-DR mAb (L243) (*e* and *f*). The percentage of cells undergoing apoptosis (annexin V-FITC-positive and PI-negative) and already dead (annexin V-FITC- and PI-positive) is indicated.

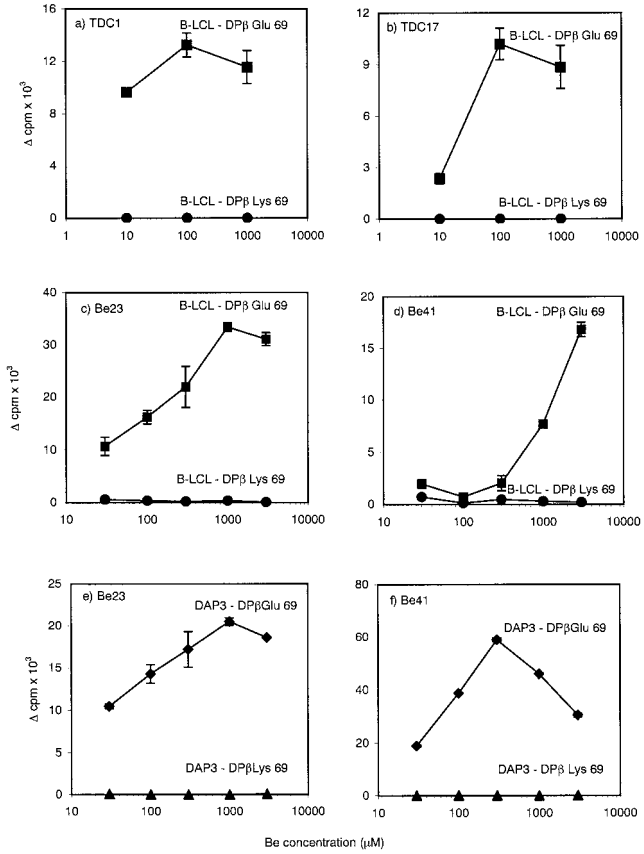

FIGURE 5. The presence of Glu at position 69 in DPβ is necessary for Be recognition by T cells. T cell clones (10^4 cells/well), TDC1 (*a*), TDC17 (*b*), Be23 (*c*), and Be41 (*d*) were cultured with 3×10^4 cells/well of Be-prepulsed B-LCLs expressing either 9036, DPB1*0201 (■) or 9013, DPB1*0402 (●), prepulsed with different doses of $BeSO_4.4H_2O$. *e* and *f*, Be 23 and Be 41, respectively, were cultured in the presence of murine DAP.3 cell transfectants expressing either 8301, DPB1*0201 (♦) or 8305, DPB1*0402 (▲). Proliferation is expressed as Δcpm.

context, we have analyzed the cytokine production profile of Be-specific T cell clones. As shown in Table III, all the Be-specific T cell clones tested produced IFN-γ and little or no IL-4. These results confirm similar data obtained using bronchoalveolar lavage (BAL) cells derived from patients with CBD (19) and suggest that the Be-specific T cell clones raised from PBMC of CBD patients are likely to be derived from pathogenic T cells. Further data supportive of this suggestion were provided by TCR gene analysis on four of the TDC clones (Table IV). Two distinguishable patterns were recognizable. T cell clones TDC1 and 30 shared a "hydrophobic-Ser-negatively charged amino acid" sequence motif at the N-terminal of CDR3 α region as well as a Thr at position 94 of the CDR3 β region, whereas TDC17 and 38 showed an identical motif "Ser-Gly-Gly-Ser" in the CDR3 region of the α-chain as well as a Ser at position 97 of the CDR3 β region. However, the most striking observation is that TDC30 displayed a CDR3 region identical, besides a conservative substitution (Val-Leu) at position 92, to a sequence previously reported as present at high frequency in the BAL of a patient with CBD (20). The associated β-chains also showed a common motif "Gly-Asp", also overrepresented in the CDR3 β region of all patients with CBD reported in the same paper (20). Moreover, the T cell clone TDC38 used the Vα22 chain as reported for the patients analyzed by Fontenot et al. (20).

Discussion

To investigate the molecular basis of HLA-DP-associated susceptibility to CBD (13), T cell clones were generated from three patients with this disease. Be recognition by all the established T cell clones was restricted by HLA-DP with Glu at residue 69, and led to the preferential secretion of IFN-γ. The DP restriction of the Be-specific clones observed here suggests strongly that the DP association in CBD reflects the role of certain alleles of DP in presentation of this metal to T cells in vivo.

There are many examples of HLA-DR and -DQ associations with resistance or susceptibility to a variety of diseases. In particular, celiac disease (CD) has been shown to be primarily associated with a pair of HLA genes: *DQA1*0501* and *DQB1*0201* (21). More recently, the same group has demonstrated that the CD-associated heterodimer is used as a restriction element in the recognition of gliadin peptides by T cells derived from the gut of CD patients (22). HLA-DP associations have also been described in autoimmune diseases, such as CD (23), insulin-dependent diabetes mellitus (24), pauciarticular juvenile rheumatoid arthritis (25–27), and juvenile ankylosing spondylitis (28). In addition, an HLA-DP association has recently been demonstrated in primary biliary cirrhosis (29), in childhood common acute lymphoblastic leukemia (30), and also in Thai individuals with enhanced vaccine-induced Ab response to a malaria sporozoite Ag (31). However, some of these associations have not been confirmed (32, 33), and in none of

Table III. *Be-specific T cell clones are mostly of Th1 phenotype*[a]

T Cell Clone	IFN-γ Concentration (pg/ml)		IL-4 Concentration (pg/ml)	
	− Be	+ Be	− Be	+ Be
TDC1	0	>1,250	0	0
TDC14	110	1,098	0.3	8.3
TDC17	41.2	>1,250	0	0
TDC30	98	>1,250	28	56
TDC38	2.8	812	0	76
Be23	4.4	51.2	0	0.2
Be41	3.1	593.8	0	0.3

[a] T cell clones were cultured with B-LCL 9036, and supernatants were harvested after 24 h. IFN-γ and IL-4 were measured using a standard ELISA kit.

these examples has the association with HLA-DP been related to the restriction element used by T cells involved in the disease. It has been shown in other studies on the association of MHC class II with chronic autoimmune disorders that single amino acid substitutions at positions 57 and 71 of HLA-DQB1 and HLA-DRB1 chains influence the susceptibility to insulin-dependent diabetes mellitus and rheumatoid arthritis, respectively (34, 35). In addition, amino acid changes in DPβ are associated with juvenile rheumatoid arthritis and CD (36–38).

Despite the evidence of DP restriction for all of the T cell clones examined, significant, although less impressive, inhibition was also seen with the anti-DRα mAb, L243. Based on the observation that cross-linking of HLA-DR on B cell lymphoma leads to apoptosis (18), we were able to demonstrate that the inhibition seen in our system was due, at least in part, to cell death of the B-LCL presenting peptide (Fig. 4). The reasons for the discrepant effect of anti-DR and anti-DP isotype-specific Abs is unclear, but may be best explained by the substantially low level of expression of the DP molecules. These observations highlight an important potential artifact in the interpretation of Ab-blocking data.

To further analyze the restriction element used by the Be-specific T cell clones, we used murine DAP.3-DP2 transfectants to present Be. Only 3 of 12 clones tested responded. One possible explanation for the failure of the murine transfectants to stimulate most of the Be-specific clones is the lack of human accessory molecules. We have shown previously that coexpression of human LFA-3 by HLA-expressing DAP.3 cells can lead to substantial augmentation of human T cell responses (39). An alternative possibility is that the T cells recognize Be bound to a particular peptide displayed by human, but not mouse cells. Species-specific differences in DR-bound peptides have been demonstrated before, in the context of T cell allorecognition using DR-expressing DAP.3 transfectants and human B-LCL (40).

In light of these considerations it remains unclear as to how Be is recognized by T cells. One possibility is that Be binds directly to the HLA-DP molecule, leading to modification of peptide binding or of DP conformation. Alternatively, Be may interact with self-peptides bound in the groove of the HLA-DP molecule, leading to the display of "altered self". These two possibilities are under investigation.

The immunogenetic analysis referred to above highlighted the importance of the Glu at position 69 in the DPβ-chain; although most commonly represented in the *DPB1*0201* allele, susceptibility was also conferred by less common alleles with this residue in the DPβ-chain (13, 41). However, this study demonstrates the functional importance of this residue by showing that presentation in the context of a Glu^{69} expressing molecule was a mandatory requirement for T cell activation as none of the clones responded to Be presented by B cells expressing *DPB1*0402*, an allele that differs only at position 69 in the DPβ-chain. The three-dimensional structure of HLA-DP has yet to be solved; however, in the predicted structure, based on the known structures of several other class II molecules, the side chain of residue 69 in the DPβ-chain points into the peptide-binding groove (42, 43). As a consequence it is likely to interact with bound peptide and be inaccessible to TCR contact.

One of the striking features of the DP association with CBD is that a single amino acid appears to determine susceptibility or resistance. Based on these observations, it is easier to envisage that the influence of Glu^{69} results from direct binding of the positively charged Be to this negatively charged site on the DPβ-chain. However, a role for HLA-DPα in Be presentation cannot be excluded, based also on the recent observation by Wang et al. that HLA-DPA1 alleles may also be related to CBD (41). Sequence homologies of the TCR α and β CDR3 regions and the extensive homology between one of the four clones and a dominant sequence detected in BAL-derived T cells from CBD patients (20) provide strong evidence of Ag-driven TCR selection. Whether this selection is driven by Be itself or by a conserved peptide to which Be is bound is unknown.

Table IV. *TCR sequences of Be-specific T cell clones*[a]

TCRAV1					
		90			
TDC1	AV12	CA	L S E A A D A G G T S Y G	K L T F G Q G	J14.3
TDC30	AV1	CA	V S D N Q G A Q	K L V F G Q G	J14.4
	AV22	*CA*	*L S D N Q G A Q*	*K L V F G*[b]	
TDC17	AV23	CA	V Q A S G G S Y	I P T F G R G	J15.3
TDC38	AV22	CA	P L R S G G S N Y	K L T F G K G	J9.3
TCRBV1					
		90			
TDC1	BV5	CA	S G T A F L	Y G Y T F G S G	J1.2
TDC30	BV12	CV	R Q T Q G D	Q P Q H F G D G	J1.5
	BV3	*CAS*	*Y G D*	*T Q Y F G*[b]	
TDC17	BV14	CA	S K L G T S T	D T Q Y F G P G	J2.3
TDC38	BV20	CA	V S V A A S K	Q Y F G P G	J2.7

[a] The sequencing of the TCR was performed using the primers indicated in *Materials and Methods.*
[b] The sequences in italics indicate the TCR analysis performed by Fontenot et al. (20).

Finally, the pattern of cytokine production by the Be-specific clones provides further evidence that these in vitro observed responses to Be reflect in vivo events in that the T cell clones appeared mostly to be of the Th1 subset that would be predicted to be pathogenic in a chronic inflammatory lung disease.

These data give a functional basis to the genetic association between CBD and HLA-DP Glu^{69} previously reported by us (13) and represent a step forward to the understanding of the pathogenesis of CBD.

Acknowledgments

We thank Ilaria Potolocchio and Luca Richeldi for HLA-DP tissue typing of controls and patients.

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