Identification of Pathogenic T Cells in Patients with Beryllium-Induced Lung Disease

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Identification of Pathogenic T Cells in Patients with Beryllium-Induced Lung Disease

Andrew P. Fontenot, Michael T. Falta, Brian M. Freed, Lee S. Newman, and Brian L. Kotzin

Chronic beryllium disease (CBD) is caused by beryllium exposure and is characterized by granulomatous inflammation with accumulation of $\text{CD}4^+$ T cells in the lungs. We analyzed TCR $\beta$-chain and $\alpha$-chain genes expressed by these $\text{CD}4^+$ T cells. In the lungs of individual patients, as well as among four of five CBD patients studied, different oligoclonal expansions within the $\text{V}3$ subset were found to express homologous or even identical CDR3 amino acid sequences. These related expansions were specific for CBD patients, were compartmentalized to lung, and persisted at high frequency in patients with active disease. Limiting dilution cloning and analysis of coexpressed TCR $\alpha$-chain genes confirmed that these TCRs were selectively expanded by a common Ag involving beryllium. Overall, homologous TCR $\beta$- and $\alpha$-chains showed identical V regions and invariant charged residues within the CDR3 but considerable variability in TCRJ usage. Remarkably, CBD patients expressing nearly identical TCRs did not share common HLA-DRB1 or DQ alleles. These results implicate particular CD4$^+$ cells in the pathogenesis of CBD and provide insight into how beryllium is recognized in human disease. The Journal of Immunology, 1999, 163: 1019–1026.

Due to its low density and high stiffness to weight ratio, beryllium continues to be utilized in the aerospace, automotive, ceramics, electronics, and defense industries (1, 2). Exposure to beryllium, which occurs almost always in the workplace, continues to be a public health concern with approximately 800,000 individuals currently at risk for developing chronic beryllium disease (CBD) (1). This disorder is estimated to develop in 1–16% of exposed individuals, depending on the nature of the exposure (3–6). The disease is characterized by granulomatous inflammation, primarily involving the lung (1). Disease severity can vary from histological abnormalities without clinical impairment or symptoms to severe lung destruction requiring transplantation. The diagnosis of CBD requires a history of exposure to beryllium, the presence of granulomatous inflammation in a biopsy specimen, and a proliferative response of blood or lung T cells to beryllium in vitro (7).

Evidence suggests that CD4$^+$ T cells are important in the immunopathogenesis of CBD (7–10). For example, sensitization to beryllium is detected by the ability of CD4$^+$ T cells to proliferate in response to beryllium sulfate (BeSO$_4$) in culture, and the development of granulomatous inflammation in the lung is associated with the accumulation of CD4$^+$ T cells in the bronchoalveolar lavage (BAL) (3, 8, 9, 11, 12). The in vitro T cell response to BeSO$_4$ requires presenting cells expressing class II MHC molecules and has been shown to be MHC restricted (9). How the TCR interacts with beryllium and the MHC remains unknown. Recent studies of BAL CD4$^+$ T cells from CBD patients showed alterations of TCR $\beta$-chain V region (V$\beta$) expression compared with peripheral blood (10). In particular, 11 of 28 CBD patients demonstrated 16 different T cell subset expansions in the BAL, and approximately one-third of these expansions expressed V$\beta$3. We hypothesized that these increased percentages of V$\beta$3$^+$ T cells in CBD patients represent clonal or oligoclonal expansions, consistent with conventional Ag stimulation.

In the present study, we analyzed the expressed TCR $\beta$-chain (TCRB) and $\alpha$-chain (TCRA) genes and specificity of expanded CD4$^+$ T cells in the lungs of CBD patients. We identified oligoclonal expansions within the V$\beta$3 subset that persisted in BAL over long periods of time but were rarely present in blood of the same patients. In addition, expressed TCRBV3 genes with identical or homologous CDR3 amino acid sequences were apparent within individual patients as well as among four of five CBD patients studied. Limiting dilution cloning and analysis of BAL CD4$^+$ T cells expressing homologous TCRB genes confirmed the coexpression of highly homologous TCR genes and strongly suggest that these clones were selected in the lung by a common Ag involving beryllium. Together, the results implicate these CD4$^+$ T cells in the pathogenesis of CBD and provide new insight into T cell recognition of beryllium and possibly other metals in human disease.

Materials and Methods

Study population

The diagnosis of CBD was established using previously defined criteria (8, 13), including the presence of granulomatous inflammation on lung biopsy, a history of exposure to beryllium, and a positive proliferative response of BAL T cells to BeSO$_4$ in vitro. Certain relevant characteristics of the patients studied are shown in Table I. A group of three healthy individuals and three sarcoidosis patients served as control subjects for the BAL studies. Informed consent was obtained from each CBD patient and control subject, and the protocol was approved by the Human Subject Institutional Review Board at the National Jewish Medical and Research Center.

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0022-1767/99/$02.00
Table 1. Characteristics of CBD patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CBD Patient 1</th>
<th>CBD Patient 2</th>
<th>CBD Patient 3</th>
<th>CBD Patient 4</th>
<th>CBD Patient 5</th>
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<td>Industry associated with Be exposure</td>
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<td>Ceramics</td>
<td>Ceramics</td>
<td>Ceramics</td>
<td>Nuclear defense</td>
</tr>
<tr>
<td>Months of Be exposure</td>
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<td>282</td>
<td>204</td>
<td>100</td>
<td>61</td>
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<tr>
<td>Corticosteroid treatment</td>
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<td>No</td>
<td>No</td>
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<td>% Vβ3+ CD4+ T cells in BAL (blood)</td>
<td>13.8 (3.9)</td>
<td>10.7 (6.2)</td>
<td>7.4 (3.5)</td>
<td>2.6 (0.8)</td>
<td>16.3 (8.0)</td>
</tr>
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<td>Class II HLA haplotype</td>
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<td>DQB1*0401</td>
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</tbody>
</table>

HLA typing was performed by standard molecular techniques at the University of Colorado Health Sciences Center Clinical Immunology and Histocompatibility Laboratory (Denver, CO).

Analysis of TCRBV3 gene expression in peripheral blood and BAL CD4+ T cells

Mononuclear cells were isolated from heparinized blood by Ficoll-Hypaque density gradient separation and from BAL as previously described (14). Peripheral blood cells from CBD patients and BAL cells from healthy control subjects were stained with FITC-labeled CD4 (Becton Dickinson, San Jose, CA), and CD4+ T cells were sorted using an Epics 751 cell sorter (Coulter, Hialeah, FL). Sorting of BAL cells from CBD and sarcoidosis control subjects for CD4+ T cells was not performed due to the predominance of CD4+ T cells in this compartment (>80% of CD4+ cells expressed CD4). However, as indicated below, all repeated and homologous TCRBV sequences in the CD4+ cells were subsequently shown to be derived from the CD4+ population because subsequent studies were performed on cells sorted for expression of both CD4 and Vβ3.

Total RNA was isolated using an acid guanidinium-phenol chloroform method, and cDNA was synthesized using 2 μg RNA/20 μl reaction. cDNA synthesis reagents included the following: reverse transcriptase (SuperScript RT; Life Technologies, Grand Island, NY), ribonuclease inhibitor (Promega, Madison, WI), and dNTPs and random hexamers (both from Pharmacia Biotech, Piscataway, NJ). One microliter of the cDNA reaction was added per each 50-μl PCR reaction mixture. PCR amplification (AmpliTaq; Perkin-Elmer, Branchburg, NJ) was performed for 35 cycles, and both 5′ and 3′ oligonucleotide primers were present at a concentration of 0.3 μM. Sequences of the 5′ TCRBV3 primer and the 3′ primer are 5′-GTCTTACGAGAGAAGAGGGCCGCT-3′ and 5′-TTCGATGACGTCAAACAC-3′, respectively. The PCR products were ligated into the pCR II TA cloning vector (Invitrogen, San Diego, CA), and the ligation products were transferred into Epicurian Coli XL-1 Blue supercompetent Escherichia coli cells (Strategene, La Jolla, CA). Colonies containing inserts were randomly selected for nucleotide sequencing. Cycle sequencing was performed using M13 reverse (5′-CAGGAAACAGCTATGAC-3′) and M13 forward (5′-CCCCAGTCACGCAGTGTAAAGGCG-3′) sequencing primers and an automated ABI 373 sequencer (Applied Biosystems, Perkin-Elmer, Foster City, CA).

Generation of T cell clones from the BAL of CBD patients

To further analyze the TCRBV3 gene repertoire and to identify the TCRA genes coexpressed by particular T cell clones, BAL mononuclear cells were stained for CD4 and Vβ3 (clone 8F10; T Cell Sciences, Cambridge, MA) (15, 16), and double-positive cells were sorted and cloned by limiting dilution. Variable numbers (1, 3, or 10 cells per well) of sorted T cells and 1 × 106 irradiated (9000 rad) TK6 feeder cells per well were cultured in 96-well microtiter plates (Falcon, Becton Dickinson Labware, Bedford, MA) in RPMI 1640 media (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (HyClone Laboratoris, Logan, UT), 20% HL-1 culture media (BioWhittaker), 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (all from Life Technologies), 0.25 μg/ml PHA (Murex Diagnostics, Dartford, England), and 10% T cell growth factor supernatant (a gift from R. J. Albertini, University of Vermont, Burlington, VT) (17). After 12–14 days of culture, T cell colonies were transferred to 1-ml cultures. Cells from confluent cultures were harvested, and total cellular RNA was isolated using a commercially available kit (RNAid PLUS; BIO 101, La Jolla, CA). cDNA was prepared, and the TCRBV3 primer and TCRCB primer as described above. Each PCR product was purified using a DNA binding membrane spin column (QIAquick PCR Purification Kit; Qiagen, Chatsworth, CA) and sequenced using a TCRBV3 sequencing primer (5′-GCAGCTCGGGTGAGGACA-3′). T cell clones were also derived by limiting dilution from peripheral blood CD4+ T cells of a normal individual after PHA stimulation using methods similar to those described above. After 14 days of culture, Vβ3+ clones were selected and transferred to 1-ml cultures in the absence of PHA. One week later, proliferation assays were performed using T cell clones (1 × 103 cells) cultured in 96-well flat-bottom microtiter plates with 1 × 105 autologous, irradiated (3000 rad) PBMCs in the presence of 1 × 10−5 M BeSO4 or 1 μg/ml PHA for 3 days. The wells were then pulsed with 1 μCi of [3H]thymidine for 16 h, and incorporation of radioactivity was determined by β-emission spectroscopy. Proliferation assays were performed in triplicate.

Beryllium-specific clones were derived after stimulation of BAL cells in 24-well plates (Costar, Cambridge, MA) in 2 ml of culture media in the presence of 1 × 10−5 M BeSO4 for 5 days. Subsequently, Ag was removed, and the lymphoblasts were expanded further in culture media containing 10% T cell growth factor as described above. After 5 days, the lymphoblasts were stained with mAbs to Vβ3 and CD4 as described above, and double-positive cells were sorted and cloned by limiting dilution with 1 × 10−5 M BeSO4 in the presence of autologous, irradiated (3000 rad) PBMCs for 12 days. Subsequently, T cell clones were further expanded in culture media containing 10% T cell growth factor supernatant (described above). T cell clones were maintained in culture by cycles of restimulation (every 2–3 wk) with 1 × 10−5 M BeSO4 in the presence of autologous PBMCs and further expansion in culture with growth factors. Proliferation assays were performed as described above in the presence or absence of BeSO4 (10−5 M or 10−4 M). Proliferation assays were performed in triplicate.

Analysis of TCRBV and TCRA gene segments expressed by the T cell clones

T cells were harvested, and total cellular RNA was extracted as described above. cDNA was synthesized, and TCRBV3 gene segments were amplified via PCR as described above. Each PCR product was purified using a DNA binding spin column (QIAquick PCR Purification Kit; Qiagen), and cycle sequencing was performed using a TCRBV3 sequencing primer (5′-GCACCTCGGGTGAGGACA-3′). RNA from T cell clones expressing the TCRBV of interest was used for anchored PCR amplification of TCRA cDNA, utilizing the 5′ RACE system (Life Technologies). In brief, TCRBV first strand cDNA was synthesized using a TCRBV-specific primer, designated GSP1 (5′-TTCAGTCTAAAACAGGAGGCTC-3′) and SuperScript II reverse transcriptase (Life Technologies). RNA was digested with RNase mixtures, and the cDNA was purified using a silica-based membrane column. A homopolymeric dC tail was added to the cDNA using dCTP and TdT. The dC-tailed cDNA was amplified for 35 cycles of PCR using the Abridged Anchor Primer and a nested TCRBV3 primer (5′-GGAGCTTACGAGGAGGACA-3′). A second round of 35 cycles of nested PCR was performed using the Abridged Universal Amplification Primer, GSP3 (5′-TCAAGAGAGATCACCAGTGTC-3′), and 3 μl of the first round PCR product. The anchored PCR product was ligated and cloned as described above. Cycle sequencing was performed using a TCRBV3 sequencing primer (5′-TGGTACACGGCAGGGTCAGG-3′). The CDR3 of each T cell clone TCRBV was verified by a TCRBV-specific PCR product was purified using a DNA binding membrane spin column (QIAquick PCR Purification Kit; Qiagen, Chatsworth, CA) and sequenced using a TCRBV3 sequencing primer (5′-GCACCTCGGGTGAGGACA-3′).
Results

Analysis of expressed TCRB genes in patients with CBD

We previously identified a subset of CBD patients with increased percentages of Vβ3+ T cells in BAL compared with blood (10). We focused the current analysis on five of these patients who were available for additional studies. Certain characteristics of these patients are shown in Table I. Four of the CBD patients were exposed to beryllium oxide in the ceramics industry, whereas patient 5 was exposed to beryllium metal in the nuclear defense industry. The duration of beryllium exposure varied from 9 to 282 mo. The diagnosis of CBD was established subsequent to the development of symptoms in patients 1, 2, and 3 while patients 4 and 5 were diagnosed via a screening program. The presence of symptoms correlated with more severe functional and radiographic abnormalities (data not shown). Although three of the five patients were being treated with corticosteroids at the time of study, all of the patients had active disease based on worsening symptoms and progressive physiologic and radiographic abnormalities. We have previously shown (10) that alterations in TCR V region expression and T cell subset expansions persist in CBD patients with active disease, despite treatment with corticosteroids. In that study, we noted that longitudinal changes in TCR V region expression did not appear to be different in patients treated with corticosteroids, compared with untreated patients.

Table I also shows the percentages of Vβ3+ CD4+ cells in the BAL and blood of these patients, as well as their HLA-DRB1, -DQB1, and -DPB1 alleles, determined by molecular typing. The relatively small Vβ3+ expansion in the lung of this individual may therefore be secondary to this baseline inefficient generation of Vβ3+ T cells. All of the CBD patients in this study possessed DPB1*0201, which has a glutamic acid (E) at position 69 and has been associated with an increased risk of developing CBD (20–22). In contrast, no common HLA-DR or -DQ alleles were shared among all individuals. Of note, CBD patient 5 shared DRB1*13 with patient 2, DRB1*0701 and three sarcoidosis patients (1) and from BAL CD4+ lymphocytes of five CBD patients (A) and from BAL CD4+ lymphocytes of three normal controls and three sarcoidosis patients (B). TCRBV3 sequences found ≥3 times or with CDR3 homology to other isolates are shown. The same CDR3 amino acid sequence is shown more than once when encoded by different TCRB nucleotide sequences. The number of identical sequences (defined at the nucleotide level) is shown over the total number of sequences analyzed for each CDR3 amino acid sequence with an aspartic acid (D) at position 96 of the BV3.1 gene (16, 18, 19). The conserved cysteine (C) of the framework 1.6 × 10−7. Clonal expansions were much less frequent and smaller in blood. For example, no repeated sequences were found in the blood Vβ3+ CD4+ subset of patient 2, and the others demonstrated only a few repeated sequences. Furthermore, although BAL samples from patients 1–4 were enriched for sets of clones with homologous TCRRs, only patient 1 had matched isolates in blood (see below). Conversely, of five clonal expansions (≥3 repeated sequences) found in the peripheral blood of these individuals, none were found in the BAL. These results clearly demonstrate oligoclonality that predominates in the BAL of these patients and shows the independent repertoires of BAL vs blood T cells in CBD patients.

The Vβ3 CDR3 expansions in individual patients as well as among four different individuals (patients 1–4) showed marked similarities (Fig. 1A). In particular, an aspartic acid (D) at position 96 of the β-chain (or at position 90 (25). These sequence data are available from GenBank under accession numbers AF078961 to AF079024.
and usually had a glycine (G) or lysine (K) at position 95 and an arginine (R) or glutamine (Q) at position 97. The relatedness of some of these sequences is also shown in Fig. 2. For example, sets of TCRs had the sequence CASSFGD, CASSLGD, or CASSSGD, usually with an arginine (R) or glutamine (Q) at the next position. Although the Vb and NDbN regions of these clones were similar, the Jb segments varied considerably. Fig. 2 also shows examples of identical b-chain amino acid sequences in the same patient and in different patients that were encoded by different nucleotide sequences, i.e., expressed by different Vb3 T cell clones. One Vb3-CASSFGD-Jb2.7 sequence was expressed in three different CBD patients. Nucleotide sequence differences among these clones precluded the possibility of a PCR contamination or artifact.

To determine whether these homologous Vb3 CDR3 sequences were associated with beryllium-induced disease, we sequenced the same TCRBV subset in the BAL of three normal individuals and three patients with sarcoidosis. Sarcoidosis served as a particularly important control disease, since the granulomatous inflammation is essentially identical to that in CBD, and sarcoidosis is also associated with the accumulation of CD4+ T cells in BAL (23, 24). Although clonal populations were identified in the BAL of both normal subjects and sarcoidosis patients, none showed the CDR3 motif found in CBD patients (Fig. 1B). Furthermore, none of the 108 and 137 sequences from normal subjects or sarcoidosis patients, respectively, showed an aspartic acid (D) at position 96 of the TCRB sequence (p < 10−10, compared with the frequency in CBD patients).

### Persistence of related Vb3+ clones in BAL

If CD4+ T cells expressing this CDR3 motif are important in the disease process, we predicted that they should persist at sites of organ involvement in patients with continued disease activity. We therefore studied BAL samples, which were available from prior time points in two patients. Table II shows serial sequence analyses in two patients studied over 3 to 5 years. All clones were detected in at least two different serial samples, and the majority of clones were detected at every time point. Some clones varied considerably in frequency. For example, in patient 1, one clonal sequence (Vb3-CASSLKD-Jb2.7) decreased from over 50% of the total sequences to 1 in 44 sequences 3 yr later. An explanation for the dramatic decrease in the frequency of this one T cell clone is unclear since patient 1 continued to require corticosteroid therapy for progressive lung dysfunction. However, over this same time period, other clones with the shared CDR3 motif increased in frequency such that the total frequency of clones with homologous TCRs at the last time point was about 50%. In patient 2, the frequency of clones with the shared CDR3 motif persisted at 40 – 50% of the Vb3+ subset over the 4-yr study period.

### TCRA gene sequences coexpressed by T cell clones with the related TCRB motif

Since recognition of Ag by the TCR involves both TCR β- and α-chains, TCRs having the same Ag specificity and related β-chain sequences should express homologous α-chain sequences. To test this prediction, Vb3+ CD4+ T cells from the BAL of two CBD
patients were cloned by limiting dilution, and TCR genes expressed by the different clones were sequenced. T cells expressing the β-chain CDR3 motif described above were found among the cloned cells at about the frequency predicted (data not shown). The TCRA expressed by these clones was determined by anchored PCR. As shown in Fig. 3, TCRA genes expressed by these T cell clones showed remarkable similarity. Thus, all utilized TCRAV22S1, out of a possible 75–100 TCRA gene segments to choose from (25). Most used AJ49, and nearly all had similar junctional sequences and length. In addition, all of the coexpressed TCRAs encoded an asparagine (N) at amino acid position 95 and all but one had a leucine (L) at position 92. In addition, two subsets of TCRAs were apparent: one encoding a serine (S) at position 93 and utilization of AJ8 or AJ11 and another encoding an arginine (R) at position 93 and utilization of AJ49.

Analysis of TCR expression after in vitro stimulation with beryllium

It was important to determine whether T cells with the related CDR3 motif could be stimulated by BeSO₄ in culture. BAL and peripheral blood T cells from CBD patients were cultured for 5 days with optimal concentrations of BeSO₄ (3, 12, 26). Consistent with previous studies (3, 12), cultures of cells from CBD patients showed vigorous proliferation, whereas cultures containing cells from control subjects showed no response and were similar to cultures without added BeSO₄ (data not shown). TCR-β-chain sequences expressed by cells before and after stimulation are shown in Fig. 4. At day 0, the frequency of TCRs with the shared CDR3 motif was 53% and 30% in patients 2 and 3, respectively. After 5 days of stimulation with BeSO₄, blasting CD4⁺ T cells predominated in the lungs of these patients and that perhaps most of the BAL Vβ3⁺ population, as well as selected cells in other BAL Vβ subsets, are capable of responding to BeSO₄ in culture.

Although peripheral blood cells from patient 2 also proliferated in culture after addition of BeSO₄, none of the sequences before or after stimulation showed the shared Vβ3 CDR3 motif. Thus, the beryllium-responsive cells in BAL vs blood appear to be derived from separate populations.

$Vβ^{3+}$ T cell clones with the shared β-chain CDR3 motif respond to beryllium in vitro

BAL cells from patient 2 were stimulated in culture in the presence of BeSO₄ and then cloned at limiting dilution in the presence of autologous, irradiated PBMCs in culture media containing BeSO₄ and T cell growth factors. After several rounds of repeated stimulation, 24 clones were expanded. Eight clones were randomly chosen for sequence analysis, and four (50%) expressed the homologous CDR3 motif as defined above. Two isolates were identical with the β-chain junctional sequence Vβ3-CASSLG-DQPQHFG-Jβ1.5 and two others were identical with the sequence Vβ3-CASSWADREAFF-Jβ1.1. In addition, all four expressed TCRAV22S1 and had the invariant asparagine (N) at position 95 and leucine (L) at position 92 of the α-chain, as shown above for other T cell clones with the conserved CDR3 motif. Four clones were analyzed for proliferative response to BeSO₄ using autologous, irradiated PBMCs as APCs, and all four clones proliferated in an Ag-specific manner (Fig. 5). One of the four clones studied, RP1, expressed the shared CDR3 motif. The frequency of this TCR β-chain sequence ranged from ~9 to 17% of the Vβ3⁺ T cells in this patient’s BAL over 4 yr. The same β-chain sequence

| Time point in Fig. 1A for patient 1. | Time point in Fig. 1A for patient 2. | Within 1–2 mo of a year. | a | b | c |

**Table II. Persistence of clonal T cell populations over time**

<table>
<thead>
<tr>
<th>Vβ3</th>
<th>NDJβN</th>
<th>Jβ</th>
<th>Initial</th>
<th>1 yr</th>
<th>2 yr</th>
<th>3 yr</th>
<th>4 yr</th>
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**Figure 3.** Deduced Vα and Vβ CDR3 sequences expressed in BAL CD4⁺ T cell clones isolated from CBD patients 2 and 3. All of the T cell clones with the Vβ3 CDR3 motif coexpressed Vα22.1. In addition, two subsets of Vαs were identified: one with a serine (S) at position 93 and utilizing Jo8, Jo11, or Jo54, and another with an arginine (R) at position 93 and utilization of Jo49. These sequence data are available from GenBank under accession numbers AF079025 to AF079040.
was found in two other T cell clones, including one from patient 3. Irrelevant \( V_\beta 3^+ \) \( CD4^+ \) T cell clones were also derived from peripheral blood \( CD4^+ \) T cells of a normal individual after stimulation with PHA and selection of clones expressing \( V_\beta 3 \). As shown in Fig. 5, these clones proliferated vigorously to PHA but all failed to respond to BeSO\(_4\) in vitro. In fact, exposure to BeSO\(_4\) appeared to inhibit proliferation in six of eight T cell clones analyzed.

### Discussion

CBD is characterized by the accumulation of \( CD4^+ \) T cells within the lung, and these cells appear to play a critical role in the development of granulomatous inflammation (7–9). However, little is known about how sensitization to beryllium occurs and why only a minority of individuals exposed to beryllium subsequently develop chronic granulomatous lung disease, usually years after leaving the workplace in which exposure occurred (4–6, 27). Identification and characterization of the pathogenic lung T cells is likely to provide important insight into these questions. In the present work, we focused on the expanded \( V_\beta 3^+ \) \( CD4^+ \) subset previously found in the BAL of some patients with CBD (10).

Sequence analysis of the TCRs expressed in the lung showed evidence of clonally expanded \( CD4^+ \) populations that expressed TCRs related to each other. These \( CD4^+ \) clones appeared to be specific for CBD patients and expressed TCRs that were shared among different patients. Cells expressing the shared CDR3 motif were present at high frequency at the site of pathology and appeared to be compartmentalized to the lungs of individual patients. The results strongly support the contention that these expanded clones, which persist in patients with active disease, represent one subset of pathogenic beryllium-responsive \( CD4^+ \) T cells.
of healthy individuals, including clones that occupied 25–30% of this subset. These clones appear to be larger than those previously identified in normal individuals (31). The clonal expansions in normal controls may represent resident lung T cell populations or result from recent exposures to foreign Ags and indicate that the presence of oligoclonal populations in lung is not necessarily evidence of pathology. In regard to the current study, the repertoire of BAL Vβ3+ T cells in CBD was found to be distinct from that in sarcoidosis patients and normal controls and, therefore, does not appear to represent the stimulation of a resident lung population. Thus, the conserved TCR β-chain CDR3 motif found in four of the five CBD patients was not seen once in the BAL of sarcoidosis patients or normal controls. Furthermore, despite a data bank of hundreds of TCRBV3 sequences in our laboratory derived from the synovial fluid and/or peripheral blood CD4+ T cells from rheumatoid arthritis patients, other patient groups, and healthy individuals, we have not previously found this related CDR3 motif.

Considering the enormous diversity of the TCR repertoire, related TCRs are predicted to occur very rarely by chance alone and almost certainly represent selection by the same or similar Ag. In four of the five CBD patients investigated, related CDR3 sequences appeared to be present in the BAL Vβ3+ subset. This CDR3 motif was not stringent in a number of positions but appeared to require an aspartic acid (D) at the 96th position of the β-chain, frequently followed by a glutamine (Q) or arginine (R) at the next position. These CDR3s also had a similar length of 7 or 8 aa. Interestingly, nearly any TCRBJ usage was allowed, which raised questions about whether these TCRs were truly related. The ultimate form of related TCRs is that which expresses different TCRB genes (i.e., different CDR3 nucleotide sequences) and therefore is derived from different T cell clones, but expresses identical TCR β-chain amino acid sequences. Examples of such clones were found in each CBD patient as well as between different patients. Selection by the same conventional Ag should also be reflected in both the TCRA and TCRB gene sequences coexpressed in related clones. Therefore, we cloned BAL CD4+ T cells by limiting dilution, identified those clones that expressed TCRB sequences predicted to be related, and sequenced the coexpressed TCR genes. Despite variation in TCRBJ usage among these T cell clones, the coexpressed TCR genes were remarkably related to each other, with identical TCRAV usage and invariant amino acids at certain nongermline-encoded positions of the α-chain CDR3. The similarity of the TCRAs provides unequivocal evidence for the relatedness of these TCRs and their selection by a common Ag.

Although conserved usage of homologous TCRs among different animals has been frequently seen after immunization with particular proteins or peptides in rodent studies, their demonstration in different patients with the same disease has been rare. In a recent study of patients with rheumatoid arthritis, despite selection of patients for particular HLA-DR4 alleles, we could not demonstrate similar TCRs in the synovial fluid among different patients (32). We have also not been able to demonstrate related TCRs in patients with sarcoidosis (30).

It is of interest that the only patient who did not share the conserved CDR3 motif was exposed to beryllium in a nuclear weapons plant whereas the other four patients were exposed in the ceramics industry. In the ceramics industry, beryllium exposure occurs in the form of beryllium oxide, as opposed to beryllium metal in the nuclear defense industry. Others have suggested that this difference, as well as differences in particle size and solubility, may influence the immune response to beryllium and possibly account for the higher frequency of disease following exposure in the ceramics industry (1). Our present results suggest that qualitative differences in CD4+ T cell recognition may also be involved. Further studies with larger numbers of patients will determine whether different TCR repertoires are related to these different types of beryllium exposures.

We also noted a remarkable separation of lung and peripheral blood CD4+ T cell repertoires in these patients. The compartmentalization in lung of a subset of beryllium-reactive cells occurred despite the presence of beryllium-responsive cells in peripheral blood in most patients with CBD (12), including the two patients tested in our study. It is well-known that beryllium-exposed individuals may demonstrate evidence of sensitization and yet display no evidence of lung pathology or physiologic abnormalities (27). Thus, in a subset of exposed individuals, peripheral blood T cells will proliferate in culture after the addition of BeSO4, but these patients may demonstrate no clinical, physiologic, or lung histopathologic abnormalities even after long-term follow-up (27). Therefore, only a subset of beryllium-reactive CD4+ T cells appears to have pathogenic potential. The present results support the contention that these clones are compartmentalized because of their different beryllium-related specificity.

We also stimulated BAL and blood cells with BeSO4 and analyzed TCR expression after T cell stimulation. Numerous studies have indicated that BeSO4 is not a polyclonal T cell activator (8, 9, 13, 26). In contrast to patients with CBD and those individuals sensitized by a previous exposure to beryllium, cells from normal individuals or patients with other diseases do not proliferate upon BeSO4 exposure. This forms the basis for the diagnostic test used in the evaluation of beryllium-exposed individuals (3, 7, 8, 12, 13). In the present work, T cells expressing the shared CDR3 motif were prevalent in the blast population after in vitro stimulation of BAL cells, indicating their ability to respond to BeSO4 in vitro. This capability to respond to beryllium in vitro was also shown for T cell clones derived from the BAL of a patient but not for irrelevant Vβ3+ T cell clones.

Previous studies have shown that T cells from patients with CBD respond to BeSO4 in culture in a class II MHC-restricted manner (9). Considering the expression of related TCRs among four different patients, it is surprising that these CBD patients demonstrated such diverse HLA-DR and -DQ types. Thus, a shared HLA-DRB1 or -DQB1 allele was not found in this group of CBD patients. Patient 5 did share HLA-DR and -DQ alleles with other CBD patients studied and yet did not demonstrate BAL Vβ3+ cells utilizing the related CDR3 motif. In contrast to HLA-DR and -DQ, all of the patients expressed DPB1*0201, which has been associated with an increased risk of developing CBD after exposure to beryllium (20–22). In preliminary studies, however, the responses to BeSO4 of lung-derived CD4+ T cell clones, including those with the shared CDR3 motif, were blocked by anti-DR mAbs and therefore appear to be restricted to HLA-DR (A. P. Fontenot, L. S. Newman, and B. L. Kotzin, unpublished observations).

The nature of the Ag that interacts with the TCR and MHC is not known. It has been hypothesized that beryllium binds to different self proteins (or peptides), which are then presented as foreign to T cells by surface class II MHC molecules. There is no direct evidence to suggest that metals (e.g., beryllium, nickel, and gold) can directly interact with the MHC molecule (33). Studies of nickel-induced contact dermatitis have demonstrated recognition of nickel in association with HLA-DRw11 (34) and suggested that nickel binds directly to the peptide component of the MHC-peptide complex via a histidine residue. The interaction of other transition metals (Co2+, Cu2+, and Zn2+) with histidine residues has also been demonstrated (35). Whether beryllium might interact with a particular exposed amino acid position of the bound peptide remains unknown. However, our finding of a CDR3 motif with
invariant charged residues at particular positions, such as the β-chain aspartic acid, is consistent with a direct interaction of the TCR with a positively charged beryllium molecule.

In summary, we believe that these studies have identified pathogenic T cells in patients with CBD and have provided insight into an unusual form of T cell recognition. Based on the repertoire of beryllium-reactive peripheral blood cells, the TCRs expressed appear to be diverse. However, only a subset of these clones, including a subset expressing homologous TCRs, appears to have pathogenic potential. Why these particular cells seem to be enriched in the lungs of patients with CBD is of critical importance in our understanding the immunopathogenesis of this disease.

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References