

Frequency of beryllium-specific, T_H1-type cytokine-expressing CD4⁺ T cells in patients with beryllium-induced disease

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Background: Beryllium sensitization is caused by exposure to beryllium in the workplace. A subset of beryllium-sensitized (BeS) subjects progress to chronic beryllium disease (CBD), a disorder characterized by a CD4⁺ T-cell alveolitis and granulomatous inflammation. Whether biomarkers are present in blood that would allow separation of CBD from beryllium sensitization without invasive pulmonary procedures is unknown.

Objective: The aim of this study was to determine whether the quantity of beryllium-specific T cells in blood of patients with CBD differs from that found in BeS subjects.

Methods: Beryllium-induced T-cell proliferation and T_H1-type cytokine secretion were determined in blood cells from 33 patients with CBD and 18 BeS subjects.

Results: We demonstrate here that patients with CBD have a significantly elevated number of IFN- γ -producing and IL-2-producing beryllium-specific CD4⁺ T cells in blood compared with both BeS and normal control subjects. In contrast, no difference in beryllium-induced proliferation of blood T cells was seen between BeS patients and patients with CBD. Compared with the blood beryllium lymphocyte proliferation test, which detected beryllium-induced proliferation in 65% of BeS patients and patients with CBD, ELISPOT analysis detected IFN- γ secretion in 80% of these subjects. Higher numbers of beryllium-specific cells in blood were also associated with the extent of alveolar inflammation, as measured by both bronchoalveolar lavage white blood cell and lymphocyte counts.

Conclusion: The frequency of beryllium-specific T cells in the blood of beryllium-exposed subjects may be a useful biomarker that helps discriminate between beryllium sensitization and progression to CBD. (*J Allergy Clin Immunol* 2005;115:1036-42.)

Key words: Human, lung, CD4-positive T lymphocyte, beryllium, autoimmunity

Abbreviations used

BAL: Bronchoalveolar lavage
BeLPT: Beryllium lymphocyte proliferation test
BeS: Beryllium-sensitized
CBD: Chronic beryllium disease
ROC: Receiver operator characteristic
SFU: Spot-forming units
SI: Stimulation index
WBC: White blood cell

Beryllium sensitization occurs in individuals exposed to beryllium in the workplace, with more than 1,000,000 US workers having been exposed and thus at risk for its development.¹⁻⁴ Beryllium-sensitized (BeS) individuals possess a beryllium-specific immune response limited to the blood and show no evidence of lung disease. Only a subset of these individuals progress to chronic beryllium disease (CBD).³ Depending on the nature of the exposure and the genetic susceptibility of the individual, it is estimated that disease develops in 1% to 16% of exposed individuals.⁵⁻⁸ CBD is characterized by granulomatous inflammation and the accumulation of beryllium-specific CD4⁺ T cells in the lung. Lung T cells are critically involved in the immunopathogenesis of disease and are composed of oligoclonal T-cell expansions that recognize beryllium in an HLA-DP-restricted manner.⁹⁻¹³ Although the vast majority of beryllium-specific CD4⁺ T cells from patients with CBD are compartmentalized to the lung, blood T cells proliferate in the presence of beryllium salts in culture.¹⁰⁻¹² The immunologic mechanisms involved in the progression from beryllium sensitization to CBD remain poorly defined.

The standard assay for documenting the presence of a beryllium-specific immune response in blood is the beryllium lymphocyte proliferation test (BeLPT).^{14,15} Because of the sensitivity of this assay, it has been used for screening and diagnosis of beryllium sensitization in the workplace and is a required component of the US Department of Energy CBD prevention program.¹⁶ However, it has been criticized because of variability in test results. In addition, the BeLPT is not used to distinguish between BeS and CBD, which currently requires invasive tests such as bronchoscopy with bronchoalveolar lavage (BAL) and lung biopsy to confirm progression to CBD. The identification of noninvasive

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TABLE I. Characteristics of BeS patients and patients with CBD*

Characteristics	BeS patients (n = 18)	Patients with CBD (n = 33)
Age, y	57 (37-87)	62 (46-81)
Gender (M/F)	15/3	29/4
Race (white/African American/Hispanic)	14/2/2	25/2/6
Smoking status (current smoker/former smoker/never smoker)	0/6/8	0/11/11
Years since diagnosis	2.6 (1.2-8.0)	7.1 (0-13.8)
Industry of exposure (nuclear/ceramic/other)	17/0/1	22/8/3
Treatment (none/prednisone/methotrexate)	18/0/0	25/7/4
BAL WBC count ×10 ⁶	18.5 (4.3-34.9)	30.5 (6.5-230)†
BAL lymphocyte count ×10 ⁶	1.3 (0.1-3.9)	4.8 (0.3-191)†
BAL BeLPT, SI	2.2 (0.9-9.3)	9.4 (1-232)†

*Data expressed as median (range).
†P < .05.

assays that detect beryllium sensitization and differentiate stages of disease would greatly advance our understanding of the immunopathogenesis of beryllium-induced disease and affect patient care.

In the current study, we analyzed beryllium-specific proliferative responses and T_H1-type cytokine secretion by CD4⁺ T cells in the blood of BeS subjects and subjects with CBD. Both patient groups compared with healthy controls were associated with markedly elevated numbers of IFN-γ-secreting and IL-2-secreting T cells specific for beryllium. Although no difference in proliferative response was seen in BeS subjects and subjects with CBD, we observed the highest frequency of antigen-specific, cytokine-secreting T cells in CBD. These results suggest that the number of circulating beryllium-specific, cytokine-secreting T cells increases with disease progression, which may prove useful as a means of separating stages of disease.

METHODS

Study population

Thirty-three patients with a diagnosis of CBD and 18 patients with a diagnosis of BeS were consecutively enrolled in this study. The diagnosis of CBD was established by using previously defined criteria, including a history of beryllium exposure, presence of an abnormal blood BeLPT on 2 separate occasions and/or BAL BeLPT on 1 occasion, and the presence of granulomatous inflammation and/or mononuclear cell infiltration on lung biopsy.^{14,17} The diagnosis of BeS was established on the basis of a history of beryllium exposure, an abnormal blood BeLPT on 2 occasions, and the absence of granulomatous inflammation or other abnormalities on lung biopsy.^{15,18} At the time of study enrollment, BeS subjects underwent bronchoscopy with BAL and transbronchial biopsy to determine whether disease progression had occurred. We purposefully enrolled some BeS subjects and subjects with CBD who repeatedly had normal or borderline blood BeLPT to evaluate the utility of the ELISPOT assay in this patient population. A total of 12 healthy nonberyllium-exposed control subjects were also enrolled.

The demographics of the BeS patients and patients with CBD are shown in Table I. Active smokers were excluded from enrollment. No difference in years since diagnosis was observed between BeS subjects and subjects with CBD, regardless of the presence or absence of beryllium-induced proliferation of blood cells. Seven patients with CBD were treated with corticosteroids, and 4 received methotrexate. Indications for treatment include severe disabling symptoms, worsening pulmonary function and/or exercise physiol-

ogy, and evidence of cor pulmonale.² Despite the presence of granulomatous inflammation and a T-cell alveolitis in the patients with CBD, no difference in pulmonary or exercise physiology was seen, with the only exception a decreased diffusing capacity for carbon monoxide in the CBD compared with the BeS group (median, 86; range, 51-121; vs median, 96; range, 73-117; P = .04). Informed consent was obtained from each patient and control subject, and the protocol was approved by the Human Subject Institutional Review Boards at the University of Colorado Health Sciences Center and National Jewish Medical and Research Center.

Lymphocyte proliferation assay

Proliferation assays were performed by using PBMCs (2.5 × 10⁵ cells/well) that were cultured for 4 to 6 days in complete culture media containing RPMI 1640 supplemented with 10% heat-inactivated human serum (Gemini Bio-Products, Woodland, Calif) with the following stimulants: medium, 2.5 μg/mL PHA, 1 × 10⁻⁴ M or 1 × 10⁻⁵ M BeSO₄. The wells were pulsed with 1 μCi [³H] thymidine for 18 hours, and incorporation of radioactivity was determined by β-emission spectroscopy. Proliferation assays were performed in quadruplicate. The data are presented as stimulation index (SI) with a positive response defined as SI ≥ 2.5.¹²

Immunofluorescence staining and analysis of intracellular cytokine expression

For stimulation of cytokine expression, 1 × 10⁶ PBMCs and 5 × 10⁵ BAL cells were exposed to medium alone, 10 ng/mL staphylococcal enterotoxin B, or 1 × 10⁻⁴ M BeSO₄ for 6 hours, with 10 μg/mL brefeldin A added after the first hour of stimulation.¹² Cells were stained with mAbs directed against CD4 and CD8 (BD Biosciences Pharmingen, San Diego, Calif) followed by fixation, permeabilization, and staining with mAbs directed against IFN-γ and/or IL-2 (Caltag, Burlingame, Calif).^{9,10,12} The lymphocyte population was identified by using forward and 90° light scatter patterns, and fluorescence intensity was analyzed by using a FACSCaliber cytometer (Becton Dickinson, San Jose, Calif) as previously described.^{9,10,12}

Analysis of IFN-γ and IL-2 production by ELISPOT assay

ELISPOT assays were performed by using plates (ImmunoSpot M200; BD Biosciences Pharmingen) that were coated with IFN-γ or IL-2 capture mAb (BD Biosciences Pharmingen) overnight and blocked with medium for 2 hours at room temperature.¹⁹ PBMCs (5 × 10⁵ cells/well) were added to wells and incubated overnight at 37°C with medium, PHA, or BeSO₄. ELISPOT assays were performed in triplicate. IFN-γ or IL-2 detection mAbs (BD Biosciences Pharmingen) were added, and spots were visualized by

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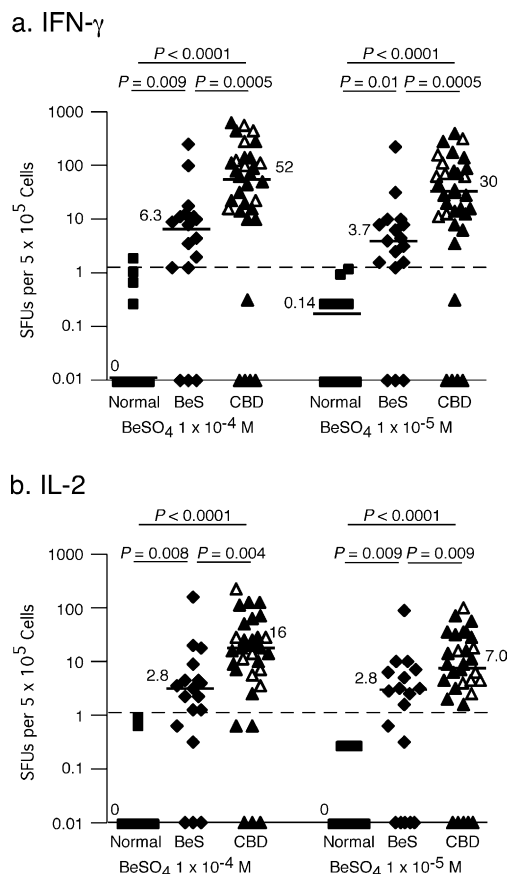


FIG 1. Frequency of beryllium-specific T cells in blood. PBMCs were evaluated by using ELISPOT analysis for IFN- γ (A) and IL-2 (B) after beryllium exposure in culture. Data are expressed as the mean SFU, and median values are indicated with solid lines. Immunosuppressant-treated subjects with CBD are indicated by open triangles. Above the dotted line represents a positive cytokine response.

avidin-horseradish peroxidase and 3-amino-9-ethylcarbazole substrate reagent (BD Biosciences Pharmingen). The ELISPOT plates were analyzed by using a CTL Immunospot Analyzer (Cellular Technology Ltd, Cleveland, Ohio), and results are reported as mean $\pm$ SD spot-forming units (SFU) per well minus background SFU.

Statistical analysis

ANOVA was used to determine whether there was a global difference between groups. After the data were checked for overall group differences, individual contrasts were calculated to compare group means of interest. Normalizing transformations were made in cases in which the data were non-Gaussian. For comparison of IL-2 versus IFN- γ expression in patients with CBD, a paired t test was used. A receiver operator characteristic (ROC) analysis is a statistical approach for evaluating the performance of new quantitative assays and was used to determine the optimal threshold value for a positive beryllium-induced T_H1-type cytokine response as measured by sensitivity and specificity (Prism 4; GraphPad Software, Inc, San Diego, Calif).²⁰ For the ROC analysis, we defined the beryllium-sensitized group (BeS and CBD) as disease-positive and the normal controls as disease-negative. A Spearman correlation was performed to analyze the association between the frequency of beryllium-specific T cells in blood and continuous variables of CBD. A P value of $<.05$ was considered statistically significant.

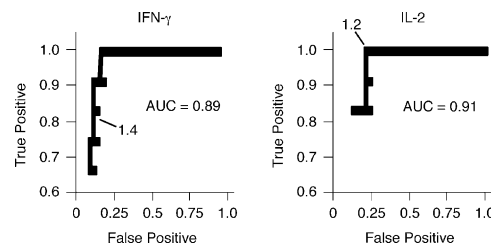


FIG 2. ROC curves for IFN- γ and IL-2 SFU in the detection of beryllium sensitization (BeS and CBD) after exposure to 1×10^{-4} M BeSO₄. The area under the curve is shown. The chosen cutoff value for each ROC curve is also shown.

RESULTS

Quantification of beryllium-specific, IFN- γ -producing PBMCs

ELISPOT assays were performed on fresh PBMCs from 12 healthy controls, 18 BeS subjects, and 33 subjects with CBD (Table I). In response to 1×10^{-4} M BeSO₄, the median number of IFN- γ -producing cells in blood was significantly higher in the patients with CBD (52 SFU; range, 0-645) compared with either BeS (6.3 SFU; range, 0-262; $P = .0005$) or normal control subjects (0.4 SFU; $P < .0001$; Fig 1, A), with similar findings seen at the lower concentration of beryllium. Within the subjects with CBD, the treated individuals (shown as open triangles in Fig 1) showed a trend toward an increased number of IFN- γ -producing cells (median, 117; range, 15-599) in blood compared with untreated subjects (median, 34; range, 0-645; $P = .07$). Removal of the treated subjects from the analysis did not alter the significance of differences between the groups. BeS subjects also possessed significantly greater numbers of IFN- γ -secreting cells compared with normal control subjects ($P = .009$ for 1×10^{-4} M BeSO₄ and $P = .01$ for 1×10^{-5} M BeSO₄).

Using a ROC curve to distinguish normal control subjects from BeS subjects and subjects with CBD, we chose a threshold of >1.4 IFN- γ SFU as an abnormal response (Fig 2). With this cutpoint, IFN- γ ELISPOT had a sensitivity of 80% and a specificity of 92%. Increasing or decreasing the threshold value resulted in a respective decrease in either the specificity or sensitivity. After stimulation with 1×10^{-4} M BeSO₄, only 1 of 12 healthy control subjects had a positive response, with 2.0 ± 2.1 (mean $\pm$ SD) SFU per 5×10^5 cells. On the other hand, 13 of 18 (72%) BeS subjects and 28 of 33 (85%) subjects with CBD demonstrated beryllium-induced IFN- γ production. Some overlap was seen between the number of IFN- γ -producing beryllium-specific T cells in BeS patients and patients with CBD. By using a ROC curve to differentiate patients with CBD from BeS subjects on the basis of the absolute number of beryllium-specific T cells in blood, a threshold value of >14 SFU for IFN- γ was chosen and had a sensitivity and specificity of 78% and 88%, respectively. As shown in Fig 1, A, 3 BeS subjects had >14 IFN- γ SFU, whereas 7 patients with CBD were below the threshold value.

Quantification of beryllium-specific, IL-2-producing PBMCs

The median number of IL-2-producing cells was significantly higher in patients with CBD (16 SFU/ 5×10^5 cells; range, 0-226) compared with either BeS (2.8 SFU; range, 0-162; $P = .004$) or normal control subjects (0 SFU; range, 0-1; $P < .0001$; Fig 1, B). Similar findings were observed at the lower concentration of beryllium. BeS subjects also possessed significantly greater numbers of IL-2-secreting cells compared with normal control subjects ($P = .008$ for 1×10^{-4} M BeSO_4 and $P = .009$ for 1×10^{-5} M BeSO_4).

We also used a ROC analysis to determine the threshold for a positive beryllium-induced IL-2 response (Fig 2). With a cutpoint of >1.2 IL-2 SFUs, IL-2 ELISPOT had a sensitivity of 78% and a specificity of 100%. Because of the lower number of IL-2-secreting T cells in the blood of patients with CBD, more overlap was observed between the number of IL-2-producing, beryllium-specific T cells in BeS subjects and subjects with CBD. Using a ROC analysis to differentiate patients with CBD from BeS subjects on the basis of the absolute number of circulating, IL-2-producing, beryllium-specific T cells, a cutoff value of >9.2 SFUs had a sensitivity and specificity of 66% and 83%, respectively.

Proliferation of PBMCs from BeS patients and patients with CBD to BeSO_4

PBMCs from the same healthy control subjects, BeS subjects, and subjects with CBD were examined for beryllium-induced proliferation at the same time as the ELISPOT assays were performed. As shown in Fig 3, none of the control subjects demonstrated a beryllium-induced proliferative response, with a median SI of 0.5 (range, 0.2-1.4) for 1×10^{-4} M BeSO_4 and 0.7 (range, 0.4-1.4) for 1×10^{-5} M BeSO_4 . All of the BeS patients and patients with CBD enrolled in this study had a positive proliferative response at some point during their course. No significant difference in beryllium-induced proliferation of PBMCs from subjects with CBD versus BeS subjects was seen for either BeSO_4 concentration (Fig 3). The median SI for PBMCs from BeS patients and patients with CBD exposed to 1×10^{-4} M BeSO_4 was 2.8 (range, 0.5-42) and 3.5 (range, 0.6-85; $P = .49$), respectively. The findings were similar when expressed as ΔCPM , with the median ΔCPM from BeS patients and patients with CBD exposed to 1×10^{-4} M BeSO_4 being 917 (range, 0-36,355) and 1514 (range, 0-20,884), respectively ($P = .85$). Overall, 10 of 18 (55%) BeS patients exhibited a positive proliferative response to 1×10^{-4} M BeSO_4 compared with 23 of 33 (70%) patients with CBD. Similar findings were seen with 1×10^{-5} M BeSO_4 .

Although this study was not designed to compare directly the sensitivity of ELISPOT analysis versus BeLPT for detection of beryllium sensitization or disease, we did note that ELISPOT analysis for IFN- γ was better able to detect the presence of beryllium-specific T cells in blood. For example, 33 of 51 (65%) subjects with CBD

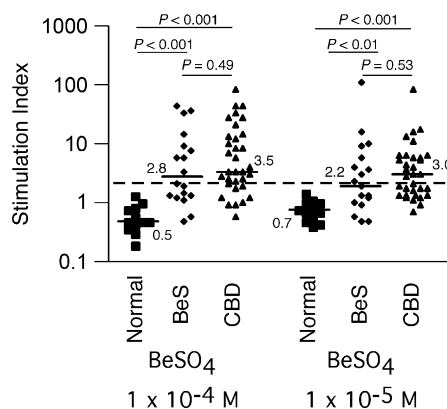


FIG 3. Beryllium-induced proliferative responses of PBMCs. The response to 1×10^{-4} M and 1×10^{-5} M BeSO_4 in culture is depicted as SI. An SI indicative of a positive response is currently defined as ≥ 2.5 (dotted line). Median values are shown and indicated with a horizontal line.

and BeS subjects had a positive beryllium-induced proliferative response in this study compared with 41 of 51 (80%) patients with a positive ELISPOT assay ($\chi^2 = 8.6$; $P = .003$). In 6 BeS subjects and 7 subjects with CBD, the ELISPOT assay detected IFN- γ -secreting and/or IL-2-secreting cells in response to beryllium exposure in culture, whereas no beryllium-induced proliferation was detected at the time of the study. Compared with BeS patients and patients with CBD with a positive blood BeLPT, similar numbers of IFN- γ -expressing and IL-2-expressing cells were seen in the blood of subjects with a negative BeLPT. For example, in patients with CBD, the median number of beryllium-induced, IFN- γ -expressing cells was 83 SFU (range, 0-645) in subjects with and 48 SFU (range, 10-112; $P = .3$) in subjects without beryllium-induced proliferation. Beryllium-induced proliferation occurred in the absence of detectable cytokine secretion in only 2 patients with CBD, whereas both ELISPOT analysis and proliferation assay were negative in 2 BeS patients and 3 patients with CBD.

Comparison of IFN- γ versus IL-2 expression on CD4^+ T cells after BeSO_4 exposure in culture

The ELISPOT analysis of blood cells suggested that a higher percentage of beryllium-specific cells from patients with CBD selectively produced IFN- γ (or lost the ability to secrete IL-2) compared with cells from BeS subjects. For example, the ratio of IFN- γ -secreting to IL-2-secreting cells determined by ELISPOT analysis was 2.7 (median; range, 0.5-167) for patients with CBD ($n = 33$) and 1.8 (median; range, 0.5-6.9) for BeS patients ($n = 18$; $P = .07$). To address this issue further, we performed intracellular cytokine staining on blood from 11 of the 33 subjects with CBD who had large enough populations ($>0.04\%$) of circulating beryllium-specific CD4^+ T cells to allow for evaluation of $\text{T}_\text{H}1$ -type cytokine expression after beryllium exposure in culture. As shown in Fig 4, A, 1.1% and 0.5% of the CD4^+ T cells from this

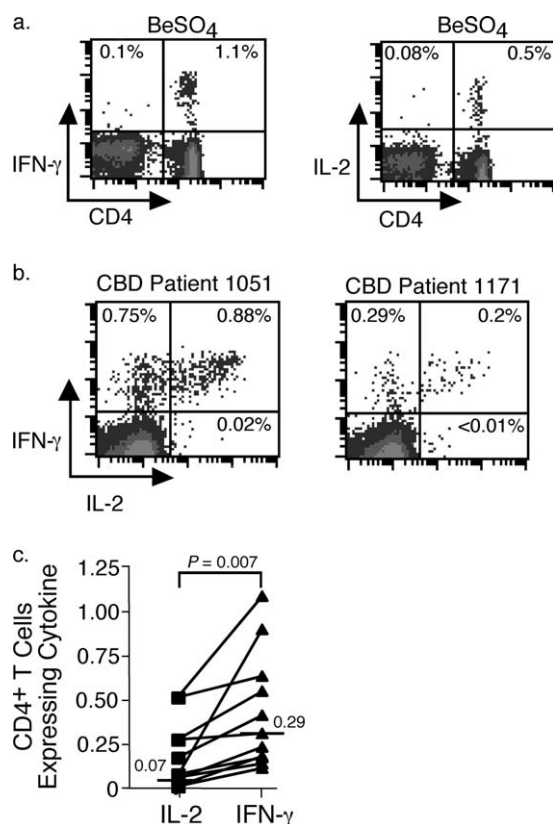


FIG 4. Intracellular expression of T_H1-type cytokines in PBMCs. **A**, Representative experiment is shown for BeSO₄-stimulated PBMCs from a patient with CBD. **B**, Analyses of IFN-γ versus IL-2 are shown for CD4⁺ T cells from 2 representative patients with CBD after BeSO₄ exposure. **C**, Frequency of IL-2-expressing and IFN-γ-expressing, beryllium-specific CD4⁺ T cells in blood (n = 11) is shown, and median value is shown.

representative subject expressed IFN-γ and IL-2, respectively, after beryllium exposure. IFN-γ and IL-2 expression in CD4⁺ T cells was equal to background levels. In the peripheral blood, the beryllium-specific CD4⁺ T cells appeared equally divided into 2 groups: T cells capable of expressing both IFN-γ and IL-2 and T cells expressing only IFN-γ (Fig 4, B). We detected almost no cells that produced IL-2 in the absence of IFN-γ expression, similar to T cells in the lung.¹² In these 11 patients with CBD, the median percentage of IFN-γ-expressing CD4⁺ T cells was 0.29% (range, 0.1% to 1.1%), compared with 0.07% for IL-2 (0% to 0.49%; $P = .007$; Fig 4, C).

Analysis of blood beryllium-specific CD4⁺ T cells in relation to clinical assessment of BeS patients and patients with CBD

We evaluated the relationship between the frequency of IFN-γ-secreting (or IL-2-secreting) T cells in blood and various markers of disease severity (Table II). No correlation was seen between the frequency of beryllium-induced IFN-γ expression in blood T cells and the duration of beryllium exposure in the workplace ($r = 0.02$; $P = .89$) or duration of disease diagnosis ($r = 0.26$;

TABLE II. Correlation of clinical variables and frequency of beryllium-specific T cells in blood of BeS patients and patients with CBD

	<i>r</i>	<i>P</i>
Beryllium exposure (y)	0.02	.89
Disease duration (y)	0.26	.15
BAL parameters		
BAL WBC count	0.41	.004
BAL lymphocyte count	0.45	.002
Exercise physiology		
Pao ₂ rest, mm Hg	0.09	.62
Pao ₂ max, mm Hg	0.05	.76
(A-a)O ₂ rest, mm Hg	-0.05	.78
(A-a)O ₂ max, mm Hg	0.004	.98

(A-a)O₂, Alveolar-arterial O₂ gradient.

$P = .15$). High numbers of beryllium-specific cells in blood were associated with the extent of alveolar inflammation as measured by the BAL total white blood cell (WBC) count ($r = 0.41$; $P = .004$) and BAL lymphocyte count ($r = 0.45$; $P = .002$). These findings suggest that IFN-γ ELISPOT analysis may be used to obtain a glimpse into the target organ without the need for invasive procedures. We observed no correlation between the number of beryllium-specific T cells in blood and pulmonary or exercise physiologic variables. This is not surprising given that the majority of CBD cases enrolled in this study were detected through workplace medical surveillance and were diagnosed at an early stage of disease with relatively normal pulmonary physiology.²¹

Longitudinal studies of the frequency of beryllium-specific T cells in blood

Nine patients with CBD and 2 BeS patients underwent repeat ELISPOT analysis at intervals ranging from 5 to 45 months after the initial evaluation (data not shown). Despite some variation in the frequency of beryllium-specific cells in blood of certain individuals over time, no consistent trend was seen, with a median of 16.7 SFU/5 × 10⁵ cells (range, 0-144) at time 0 compared with 6.0 SFU (range, 0-105; $P = .2$) at the subsequent evaluation. Because of the small number of patients and the short duration of follow-up, we were not able to analyze changes related to progression in clinical status.

DISCUSSION

Quantification of antigen-specific CD4⁺ T cells in human disease is a difficult task because of the low frequency of these cells circulating in blood and the absence of a known stimulating antigen in most cases. With the presence of a known antigenic stimulus (ie, beryllium), CBD forms an ideal system in which to define the quantity of antigen-specific T cells in blood. This disorder is mediated by CD4⁺ T cells, and these cells recognize beryllium in association with certain HLA-DP molecules.¹¹ After antigen recognition, the pathogenic

T cells proliferate and secrete T_H1 -type cytokines.^{1,22} The vast majority of beryllium-specific effector $CD4^+$ T cells are compartmentalized in the lung, with as many as 30% of the BAL $CD4^+$ T cells producing IFN- γ after beryllium exposure.¹² However, beryllium-specific $CD4^+$ T cells are present within the circulating pool because these cells proliferate in the presence of beryllium salts in culture.^{10,14,18} Using ELISPOT analysis, our results demonstrate that patients with CBD have markedly increased numbers of beryllium-specific, cytokine-secreting T cells in blood compared with BeS patients, suggesting that the quantity of beryllium-specific $CD4^+$ cells in blood could be developed as a biomarker for determining stage of disease.

All BeS patients and patients with CBD enrolled in this study had 2 documented abnormal BeLPTs at some point during the course of their disease. Despite this, $\frac{1}{3}$ of the BeS subjects and subjects with CBD enrolled in this study had a negative blood BeLPT. We purposely enrolled all study subjects who had a confirmed diagnosis of BeS or CBD, including subjects who had negative or borderline BeLPTs at the time of our analysis, to evaluate the utility of the ELISPOT assay in this population. The sensitivity of the BeLPT (65%) in this study was lower than previously reported^{5,6,15} but similar to that recently reported by Maier et al.²³ Of the 33 patients with CBD enrolled in this study, 85% showed granulomatous inflammation, and 97% had proliferation of BAL cells in response to beryllium salts. Thus, it is unlikely that the decreased sensitivity of the blood BeLPT is a result of misdiagnosis. Whether the beryllium-induced proliferative response fluctuates over time in certain individuals remains unknown. Another possibility is that the beryllium-specific $CD4^+$ T cells lose proliferative capacity while retaining the ability to secrete IFN- γ on antigen exposure. Regardless, it is important to emphasize that the sensitivity and specificity of the IFN- γ ELISPOT was similar to that of the blood BeLPT (sensitivity, 89%; specificity, 95% to 100%) reported in previous studies.^{5,6,15}

Despite significantly increased numbers of beryllium-specific T cells in the blood of patients with CBD compared with BeS subjects, we observed no difference in beryllium-induced T-cell proliferation, suggesting the possibility of a proliferative defect in blood T cells from patients with CBD. We have previously shown that certain beryllium-specific $CD4^+$ T cells in the lung proliferate poorly despite the ability to secrete IFN- γ and TNF- α .¹² These terminally differentiated lung effector memory cells appear to lose the ability to proliferate, yet are poised to secrete T_H1 -type cytokines on beryllium exposure. In preliminary experiments, we found that the majority of beryllium-specific $CD4^+$ T cells in blood also express an effector memory phenotype (A. Fontenot et al, unpublished data, June 2004). Previous studies have shown that effector memory T cells produce more IFN- γ than IL-2 and have a decreased proliferative potential compared with central memory cells.^{24,25} In this study, we identified 2 populations of beryllium-specific T cells in blood: (1)

IL-2/IFN- γ -secreting cells and (2) IFN- γ -secreting cells. We did not identify cells capable of secreting only IL-2. These observations help explain the detection of IFN- γ -secreting beryllium-specific T cells in 13 BeS patients and patients with CBD who did not show a beryllium-induced proliferative response. Taken together, these findings suggest that chronic antigen exposure, such as beryllium, may push $CD4^+$ T cells from an IL-2-producing (proliferating) T-cell phenotype to IFN- γ -secreting (poorly proliferating) T cells.

By using MHC class I tetramers containing a peptide mimotope designated NRP for which the endogenous peptide remains unknown, Trudeau et al²⁶ showed that the development of autoimmune diabetes in nonobese diabetic mice could be predicted on the basis of the frequency of islet β -cell-specific $CD8^+$ T cells in blood. Despite the fact that T cells have long been implicated in the pathogenesis of most autoimmune diseases, this was the first demonstration of a correlation between the frequency of antigen-specific T cells in blood and the onset of disease.²⁶ At the time of our PBMC analysis, 3 BeS subjects had progressed to CBD on the basis of the presence of granulomas in lung biopsy samples. In response to 1×10^{-4} M $BeSO_4$, these 3 individuals had significantly more IFN- γ -producing and IL-2-producing cells compared with the majority of BeS patients evaluated in this study. In addition, the 1 BeS subject with a beryllium-specific immune response present in both blood and BAL had the greatest number of IFN- γ -producing and IL-2-producing cells in response to 1×10^{-4} M $BeSO_4$ of all of the BeS subjects. It is possible that this subject had already progressed to CBD despite the absence of granulomatous inflammation on lung biopsy. Although progression from sensitization to disease occurred in a small number of BeS subjects, these findings suggest that the transition from beryllium sensitization to CBD may be associated with an increased number of beryllium-specific T_H1 -secreting T cells circulating in the blood.

An important unanswered question in human disease is whether the frequency of antigen-specific T cells in blood remains stable or fluctuates over time. In nonobese diabetic mice, the populations of autoantigen-specific $CD8^+$ T cells appeared in distinct cycles before manifestation of diabetes.²⁶ Our previous studies have shown that both T cell receptor variable region repertoire alterations and the frequency of beryllium-specific $CD4^+$ T cells in lung remain stable over time.^{9,12} In the current study, we found no association between the duration of disease and the frequency of beryllium-specific $CD4^+$ T cells in blood in individuals who did not change their disease classification. One possible reason for this may relate to the presence of beryllium in the lung decades after exposure has ceased, which may provide a continued antigenic stimulus. Thus, CBD serves as a model of persistent antigen exposure, suggesting the possibility that the quantity of beryllium-responsive $CD4^+$ T cells in blood may remain stable over time. However, it remains possible that the frequency of beryllium-specific $CD4^+$ T cells in

blood fluctuates over the course of disease, with a higher frequency of circulating antigen-specific cells during periods of worsening lung inflammation. In this study, we did observe a significant correlation between the frequency of beryllium-specific cells in blood of patients with CBD and the degree of lung inflammation as measured by the number of WBCs, in particular lymphocytes, in the BAL. However, we were unable to correlate the frequency of beryllium-specific T cells in blood with any clinical marker of disease severity.

Beryllium-specific CD4⁺ T cell function is currently assessed by using the BeLPT.² However, this assay requires that beryllium-specific cells maintain the ability to proliferate in culture after antigen stimulation, and it does not allow for a precise quantification of the actual frequencies of responding cells. Additional advantages of ELISPOT analysis include a shorter duration (24 hours vs 6 days) and lack of the need for radioactivity. As has been noted for other systems such as the immune response to malaria²⁷ and tuberculosis,²⁸ ELISPOT assay may preferentially identify an effector memory T-cell subset, whereas the BeLPT identifies a central memory subset of beryllium-responsive cells. If further studies confirm that the majority of beryllium-specific CD4⁺ T cells in blood express an effector memory T-cell phenotype, the ELISPOT assay may give a more accurate estimation of the degree of beryllium sensitization in the workplace.

In summary, our findings suggest the need for a prospective study comparing ELISPOT and proliferation assays in a larger patient cohort to determine which assay is superior for the identification of beryllium sensitization in the workplace. The cross-sectional differences in number of cytokine-secreting cells between BeS patients and patients with CBD provide a basis for future studies to test whether ELISPOT analysis can predict progression from beryllium sensitization to CBD and disease severity.

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