

Case Report

***Nocardia otitidiscaviarum* meningitis in a diffuse large B-cell lymphoma patient with CD4-positive lymphocytopenia and persistent oligoclonal CD8-positive lymphocytes in the peripheral blood**

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Abstract: Nocardiosis, sometimes presenting with multiple granulomatous lesions, is a rare opportunistic infection occurring in immunocompromised patients. However, its immunological features remain largely unaddressed. We investigated the immunological characteristics of human nocardiosis and examined the component cells of the granulomatous lesions. A 66-year-old man with diffuse large B-cell lymphoma presented with fever and multiple nodules in the lung during chemotherapy. The blood culture formed white colonies, but their characterization was difficult by routine microbiological laboratory methods. Matrix-assisted laser desorption ionization-time of flight mass spectrometry identified the colonies as *Nocardia otitidiscaviarum*. Meanwhile, the patient suddenly experienced an epileptic seizure without a brain abscess. His cerebrospinal fluid (CSF) showed neutrophilic pleocytosis (108/mm³). The conventional agar culturing failed to isolate colonies, but culturing with brain-heart infusion agar generated colonies. These colonies were completely concordant with those from the blood, as confirmed by 16S rRNA gene sequencing. Therefore, the patient had developed meningitis through sepsis induced by *N. otitidiscaviarum*. His CD4-positive T-lymphocyte counts were low, and oligoclonal CD8-positive $\alpha\beta$ T-lymphocytes were present in the blood prior to the first and after three cycles of chemotherapy. He had bone marrow granulomatous lesions comprising lymphoma and CD8-positive $\alpha\beta$ T-cells. Treatment with sulfamethoxazole/trimethoprim relieved all of his symptoms. The combined analysis by microbiological and molecular methods determined the cause of his epileptic seizure. His immunological characteristics, including low CD4-positive or CD8-positive $\alpha\beta$ T-lymphocytes, may have contributed to the unusual clinical presentations by *N. otitidiscaviarum*, which rarely involves the central nervous system.

Keywords: *Nocardia otitidiscaviarum*, meningitis, idiopathic CD4-positive T-lymphocytopenia, oligoclonal CD8-positive T-lymphocytes, granulomatous lesions

Introduction

Nocardiosis predominantly occurs in immunocompromised patients with impaired cell-mediated immunity, such as idiopathic CD4-positive (+) T-lymphocytopenia (ICL), as characterized by the United States Centers for Disease Control [1, 2]. However, ICL is still a condition with a largely unknown etiology. Animal models have suggested the immunological agents responsible for susceptibility to *Nocardia* infection and its underlying mechanism, whereas the human

immunological features of nocardiosis remained largely unaddressed [3-5].

Recent studies have indicated that T-cell-mediated immunity impairment is a common feature in patients with malignant lymphomas, which include a lack of diversity in the T-cell receptor (TCR) repertoire and/or disproportion in the TCR $\alpha\beta$ sub-families [6-8]. However, little is known about the characteristics of T-cells in malignant lymphoma patients complicated with *Nocardia* infections [9].

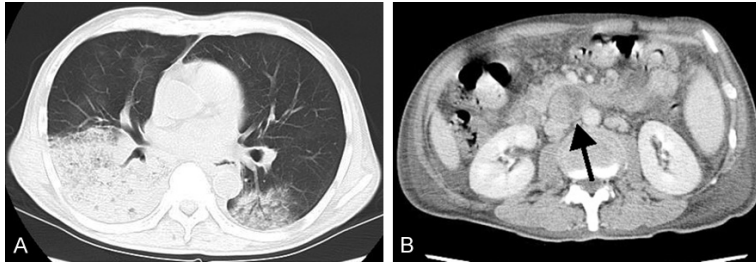


Figure 1. Computed tomography scan of the patient at the first hospitalization. A. Ground-glass opacities of both lungs. B. An enlarged lymph node with low-contrast lesions in the para-aortic area (arrow).

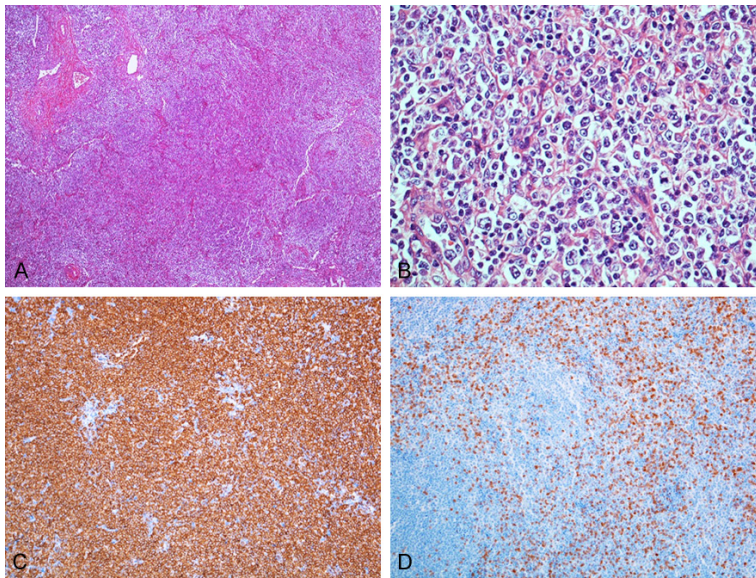


Figure 2. Pathological findings of a biopsied lymph node from the left side of the neck. A. Loss of normal lymph node architecture (hematoxylin-eosin stain; 40-fold magnification). B. Diffuse infiltration of intermediate to large lymphoma cells (hematoxylin-eosin stain at 400-fold magnification). C. Diffusely infiltrated cells were positive for CD20 (brown-colored) (immunohistochemical stain at 100-fold magnification). D. Diffusely infiltrated cells were negative for CD3 (immunohistochemical stain at 100-fold magnification).

Nocardiosis sometimes generates abscesses or granulomatous lesions in the lung, subcutaneous tissue, or lymph nodes. However, its precise mechanism still remains unclear. Recent experimental studies with models of granulomatous pulmonary nocardiosis revealed that *Nocardia* micro-colonies developed pulmonary granulomas, and TCR $\gamma\delta$ -lymphocytes and IL-17 play a critical role in controlling pulmonary nocardiosis. The granulomatous lesions associated with human nocardiosis have not been histologically analyzed, and bone marrow granulomatous lesions have never been reported.

Recently, a number of identified *Nocardia* species were reclassified by 16S rRNA gene se-

quencing, and some species were shown to be associated with human infections [10]. *Nocardia otitidiscaviarum*, a rare cause of human nocardia infections, is infrequently involved in the central nervous system (CNS) and has low pathogenicity [2, 11]. These characteristics distinguish it from other *Nocardia* species, resulting in the unusual clinical presentations and laboratory data, and it being referred to as the great imitator. Consequently, the detection of *N. otitidiscaviarum* involves a number of challenging problems, including isolation and culture methods, especially when solely using conventional microbiological methods. Thus, modern methods, such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing, have been applied for the accurate and rapid identification of *Nocardia* species [12].

Here, we describe for the first time a malignant lymphoma patient who experienced an epileptic seizure associated with meningitis caused by *N. otitidiscaviarum*, which was precisely identified by a combination of microbiological and molecular methods, including MALDI-TOF-MS and 16S rRNA sequencing.

We investigated his immunological characteristics, and analyzed the bone marrow granulomatous lesions by immunohistochemistry.

Materials and methods

A 66-year-old Japanese man with fever and vomiting arrived at the emergency room of our hospital. His past medical history included a learning disability and epilepsy in early life, but he had not experienced any epileptic seizures for over two decades, even without drug administration.

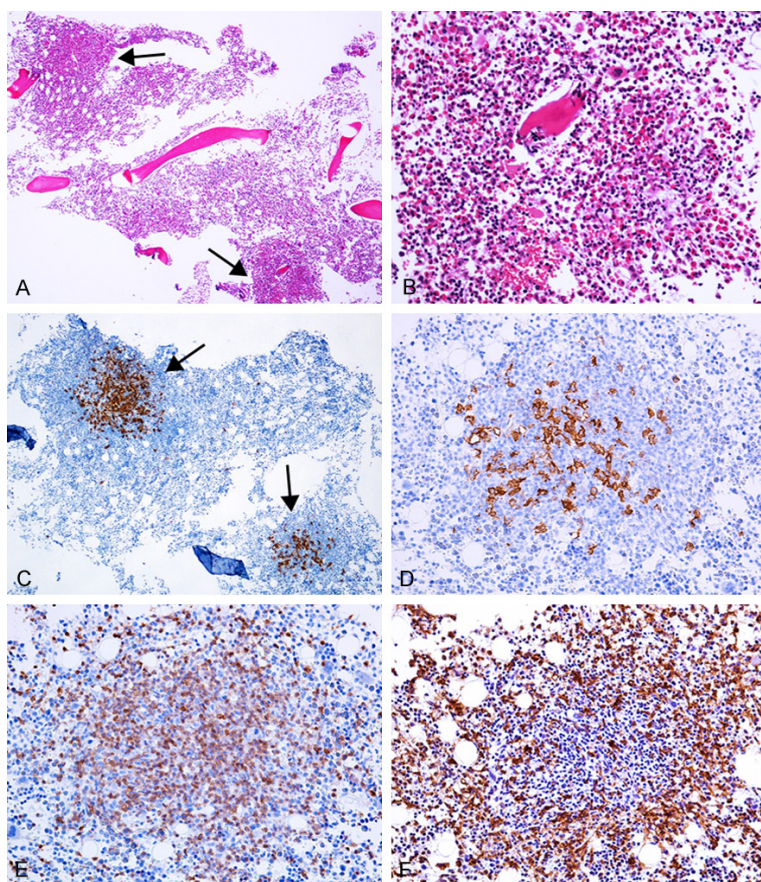


Figure 3. Pathological findings of biopsied bone marrow tissue. A. Several granulomatous lesions in the bone marrow (arrows) (hematoxylin-eosin stain at 40-fold magnification). B. Granulomatous lesion containing numerous eosinophils (hematoxylin-eosin stain at 200-fold magnification). C. CD20-positive cells selectively localized in granulomatous lesions (arrows) (immunohistochemical staining at 40-fold magnification). D. Several CD20-positive large cells in a granulomatous lesion (immunohistochemical staining at 200-fold magnification). E. Numerous CD3-positive small lymphocytes in a granulomatous lesion (immunohistochemical staining at 200-fold magnification). F. Numerous CD68-positive cells were localized at the periphery of a granulomatous lesion (immunohistochemical staining at 200-fold magnification).

He presented with fever, which had persisted for 7-days prior to his admission. His blood pressure was 94/61 mmHg; his pulse rate was 95 beats/min; his respiratory rate was 18/min; and his oxygen saturation degree was 90%. Auscultation revealed right basal crepitation and normal heart sound. He had enlarged lymph nodes on the left side of the neck. The laboratory findings were: hemoglobin level, 8.9 g/dL; white blood cell count, 5400/mm³ (neutrophils, 84.4%; lymphocytes, 11.7%); platelet count, 19.4 × 10⁴/mm³; and C-reactive protein concentration, 3.8 mg/dL. The serum liver enzyme levels were within normal limits. Tests for human immunodeficiency virus antibody,

hepatitis C virus antibody, and hepatitis B virus surface antigen were all negative. *Candida* antigen, *Aspergillus* antigen, β-D glucan, and *Cytomegalovirus* antigenemia tests were all negative.

A computed tomography scan (CT) revealed moderate ground-glass opacities in both lungs, splenomegaly, multiple enlarged lymph nodes, and no abnormal findings in the brain (**Figure 1**). Cultures of his blood and sputum isolated *Streptococcus pneumoniae*. Together, these findings suggested that the patient had malignant lymphoma as well as sepsis and pneumonia caused by *Streptococcus pneumoniae*. The patient's soluble interleukin-2 receptor concentration was 4,790 U/mL (normal: 124-466). He was treated with intravenous sulbactam for 10 days, which relieved his symptoms, and his laboratory data rapidly returned to normal levels.

A biopsy of the left-neck lymph node was performed, which revealed diffuse infiltration of medium to large cells, which were positive for CD20, CD79a and bcl-2, and negative for CD3, CD5, CD10 and CD30

(**Figure 2**). These findings led to a diagnosis of diffuse large B-cell lymphoma (DLBCL). An immunohistochemical examination of the bone marrow disclosed granulomatous lesions, comprising several CD20+ large cells, many CD3+ medium cells, and eosinophils in the central region surrounded by CD68+ cells (**Figure 3**). CD21- and CD23-signals were negative in these granulomatous lesions. A gene rearrangement study by polymerase chain reaction (PCR) revealed the presence of oligoclonal lymphocytes with rearrangements of the T-cell receptor (TCR) β variable-joining or diversity-joining chains in both the peripheral blood and bone marrow cells (**Supplementary Figure 1**). Flow

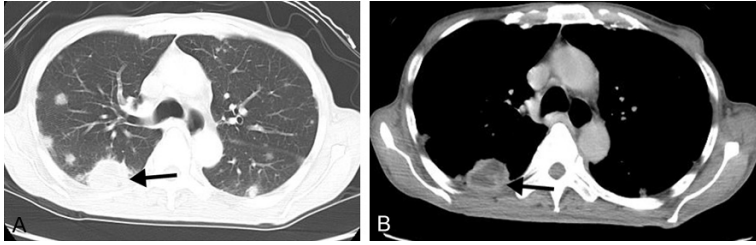


Figure 4. Computed tomography scan at the second admission. A. Multiple nodular lesions in the lung (arrow). B. Fluid collection, which was suggested to be abscess formation, in a nodular lesion (arrow).

cytometry showed the disproportion of TCR $\alpha\beta$ lymphocytes (87.3%: 611/mm³) (normal: 1416 $\pm$ 267/mm³) and TCR $\gamma\delta$ lymphocytes (4.9%: 34/mm³) (normal: 110 $\pm$ 53/mm³) in the peripheral blood. The CD4+ T-lymphocyte counts were 125/mm³, the CD4+/CD8+ ratio was 0.2, and the CD19+ B-lymphocytes were deficient (Supplementary Figure 2). T-lymphocytes with the rearranged TCR γ or δ chain were not detected in the peripheral blood by a Southern blotting analysis. Together, these results indicated that the oligoclonal T-lymphocytes with TCR β rearrangements were mostly characterized as CD8+ $\alpha\beta$ T-lymphocytes. His peripheral blood lymphocytes contained a few azurophilic granules (Supplementary Figure 3).

The third course of R-CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone, and rituximab) was started, and 7 days later, the patient complained of high-fever and chest pain. A CT scan revealed multiple nodular lesions in both lungs (Figure 4). Collectively, these findings suggested multiple abscesses in both lungs. Tests for β -D glucan or *Aspergillus* antigen in the blood, cytomegalovirus-antigenemia, and Legionella antigen in the urine were negative. White and roughened colonies that were Gram-positive and filamentous were isolated from the blood after a 5 day incubation. However, conventional bacteriological analyses failed to characterize the isolated colonies, which were finally suggested to be *N. otitidiscaviarum* by the MALDI-TOF MS analysis. The isolated strain was sensitive to linezolid and sulfamethoxazole/trimethoprim (ST), as identified by an antimicrobial susceptibility test (Supplementary Table 1).

During these identifications, the patient suddenly became fragile, and suffered an epileptic seizure. A CT scan revealed no abnormal find-

ings in the brain. An examination of the cerebrospinal fluid (CSF) showed the following findings: white blood cell count, 108/mm³ (neutrophils, 97%; lymphocytes, 3%); protein concentration, 98 mg/dL; and glucose concentration, 33 mg/dL (blood glucose, 90 mg/dL). Gram stains and smears for acid-fast bacilli were negative. Five examinations of the CSF failed to isolate any

pathogen, using routine blood agar and/or BTB agar plates.

We tried other methods, including broad-range PCR for common 16S rRNAs (Supplementary Figure 4) and cultures on brain-heart infusion agar (BHI-agar) plates containing 1% glucose and glycerol. These cultures generated brown-colored colonies from the CSF, whereas the PCR amplified no pathogens. The strain isolated from the CSF was identified as a *N. otitidiscaviarum*-type strain (Genbank, AF430067), and shared 100% similarity to the 16S rRNA sequences of the blood strain by a 16S rRNA sequencing analysis (Supplementary Figure 5). Together, these findings indicated that the patient developed meningitis with an epileptic seizure through septicemia induced by the same *N. otitidiscaviarum*. The two strains had identical antimicrobial susceptibilities (Supplementary Table 1).

Treatment with ST 960 mg thrice daily was administered via a stomach tube, in addition to a linezolid treatment. Thereafter, the patient gradually recovered from consciousness disturbances, including the epileptic seizure. Surprisingly, the oligoclonal T-lymphocytes with identical TCR rearranged bands persisted in his peripheral blood, even after three cycles of R-CHOP (Supplementary Figure 1).

We retrospectively examined whether *N. otitidiscaviarum* was present in the first specimens, including the bone marrow cells and the biopsied-lymph node, using a PCR method. Conclusively, the PCR did not detect the *N. otitidiscaviarum* gene.

Discussion

Nocardiosis has been frequently reported in patients with cell-mediated immunodeficient

states, such as ICL. In contrast, secondary immunodeficient states, such as various malignancies and their related medications, transiently decrease CD4⁺ lymphocytes while the normal CD4⁺/CD8⁺ ratio is maintained [1]. The present patient showed not only low-CD4⁺, B-cell, and NK-cell counts but also a low CD4⁺/CD8⁺ ratio, prior to the first chemotherapy. In some ICL patients, low-B-cell and/or NK-cell numbers have also been observed [1]. Collectively, this patient met the criteria for ICL, rather than malignancy or its chemotherapy-related secondary immunodeficiency.

In previous animal model studies, TCR $\gamma\delta$ -lymphocytes as well as TCR $\alpha\beta$ -lymphocytes inhibited *Nocardia* invasion and growth in the lungs [3, 13]. A recent human study showed that TCR $\gamma\delta$ -lymphocyte counts were low in the peripheral blood of ICL patients [1]. These findings may combine a low CD4 count with a low TCR $\gamma\delta$ -lymphocyte count into a human *Nocardia* infection risk, as found in our case. This is the first report describing the TCR $\gamma\delta$ -lymphocyte proportion in human nocardiosis.

In contrast, several reports have shown that oligoclonal expansions of TCR $\alpha\beta$ -lymphocytes were detected in the peripheral blood of DLBCL patients, and suggested that this expansion was due to the restriction of the TCR β -repertoires by the putative immune response to unidentified lymphoma-associated antigens [7, 8]. These TCR-repertoire restrictions are considered to be a cause of the T-cell-mediated immune dysfunction in DLBCL [6]. In the present patient, the oligoclonal TCR8⁺ $\alpha\beta$ T-lymphocytes persisted in the peripheral blood throughout the therapeutic process. The presence of TCR-repertoire restrictions may be a risk marker for *Nocardia* development in DLBCL patients.

Nocardiosis occasionally causes granulomatous lesions in several tissues. However, a previous study showed that the coincidence of both granulomatous lesions and malignant lymphoma is a rare pathologic finding, and suggested that CD8⁺ lymphocytosis and disproportions of T-cells might contribute to granulomatous lesion formation in malignant lymphoma patients [14]. In general, granulomatous lesions in bone marrow were most commonly induced by infections, followed by sarcoidosis, malignancy, and drugs [15]. In this

case, the bone marrow granulomatous lesions comprised lymphoma cells, CD8⁺ $\alpha\beta$ T-lymphocytes, eosinophils, and CD68⁺ macrophages. The eosinophilic accumulation in the granulomatous lesions may be induced by eosinophil-autoactivation against lymphoma cells and/or interactions with T-lymphocytes [16]. Extensive examinations, including a PCR analysis, did not detect *Nocardia* bodies in the bone marrow. Collectively, in the present patient, the bone marrow granulomatous lesions may be associated with oligoclonal CD8⁺ $\alpha\beta$ T-lymphocytes, possibly against lymphoma cells. This is the first report of the presence of bone marrow granulomatous lesions and the examination of their component cells in human nocardiosis.

The patient presented with an epileptic seizure as the initial symptom caused by *N. otitidiscaviarum* meningitis, without a brain abscess. *Nocardia otitidiscaviarum* produces fewer human infections than the other nocardia species, and its CNS involvement is extremely rare [2, 11]. Brain abscesses caused by *Nocardia* frequently induce consciousness disturbances, including epilepsy [17]. *Nocardia* meningitis mostly presents with the secondary involvement of a brain abscess or its rupture into ventricles. The primary *Nocardia* meningitis without predisposing conditions is quite rare [18]. An epileptic seizure as the initial symptom caused by *Nocardia* meningitis has never been reported. Our patient had a characteristic medical history of epilepsy in his early life, and this factor may have enhanced his epileptogenic sensitivity to stimulation with *Nocardia* meningitis.

The CSF findings, such as the slight neutrophilic pleocytosis, were inconsistent with the typical bacterial meningitis findings caused by bacteria such as *Streptococcus pneumoniae* [19]. A previous study showed that the routine CSF Gram-staining, which is valuable for detecting common bacterial meningitis, was not useful for the detection of *Nocardia* meningitis [18]. The isolation of *Nocardia* from blood or CSF is difficult because *Nocardia* grows slowly and has an inert metabolism *in vitro*, and versatile and sensitive media for its detection remain undetermined. Conventional bacteriological media, such as sheep blood agar and chocolate agar, may not be appropriate for its isolation [17]. In the present case, the presence of *Nocardia* in the blood was detected with a

sheep blood agar plate, whereas that in the CSF was detected only on a BHI-agar plate. Collectively, the CSF may contain lower amounts of *N. otitidiscaviarum* than the blood, and/or BHI-agar plates may be more suitable for the isolation of this *Nocardia* strain. Therefore, physicians should ask the laboratory microbiological technicians to carefully culture the CSF or blood for a longer time and/or with a variety of media or plates including non-selective media, whenever unusual infectious symptoms appear in immunocompromised hosts.

Advanced molecular methods, such as 16S rRNA sequencing and MALDI-TOF MS analysis, are useful and valuable tools in research and clinical laboratories to identify and study the taxonomy of *Nocardia* species [2, 12, 20]. The sensitivity of these methods of pathogen-detection is considered to be superior to that of conventional microbiological methods [12]. However, in our case, only the elaborated classical culture method isolated *Nocardia* from the patient's CSF. The MALDI-TOF MS may be the most appropriate detection method for abundant or isolated organisms. Finally, we emphasize that microbiological procedures as well as molecular methods remain essential and foremost for identification, and that both are complementary to each other.

We describe a DLBCL patient with the unusual initial symptom of epileptic seizure caused by *N. otitidiscaviarum*, characterized by lower pathogenicity and rare CNS involvement. His precise diagnosis was based on a combination of molecular and microbiological culture methods. Persistent oligoclonal CD8+ $\alpha\beta$ T-lymphocytes, possibly associated with DLBCL, and the low-CD4+ lymphocyte count may have impaired the patient's cellular immunity, and induced granulomatous lesions in his bone marrow. These findings provide novel basic insights for potential diagnostic and pathophysiologic avenues in *Nocardia* infections.

Acknowledgements

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Disclosure of conflict of interest

None.

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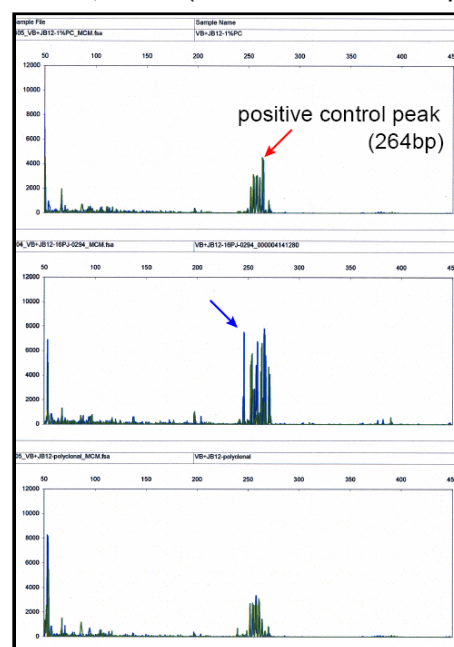
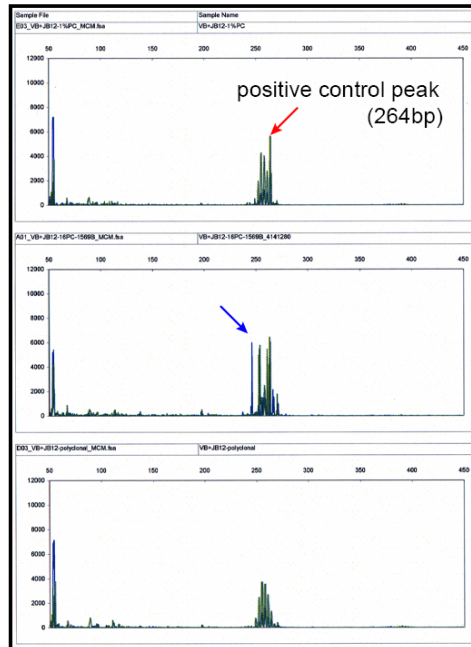
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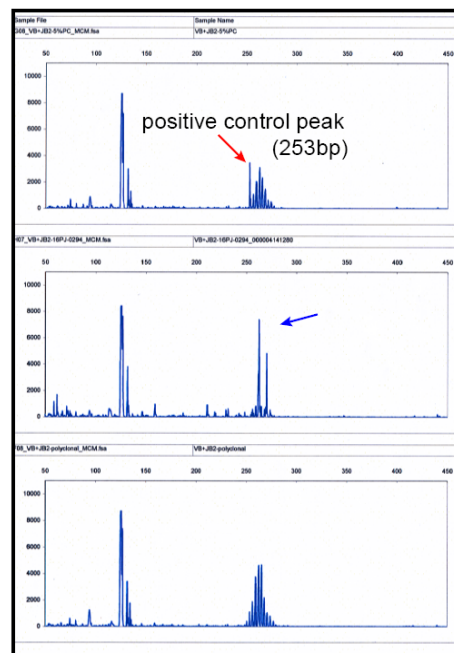
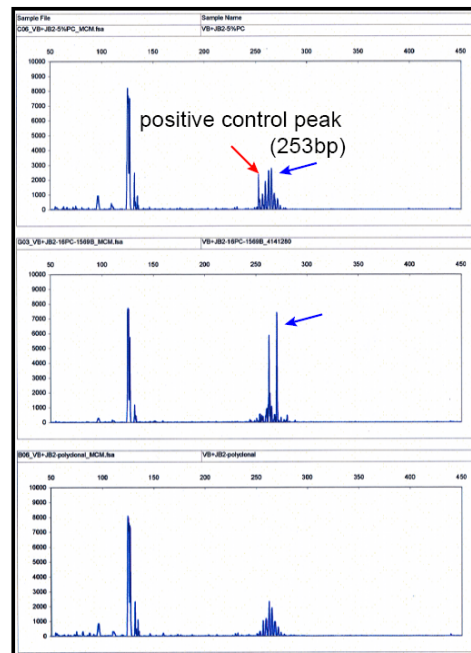
Nocardia meningitis in diffuse large B-cell lymphoma

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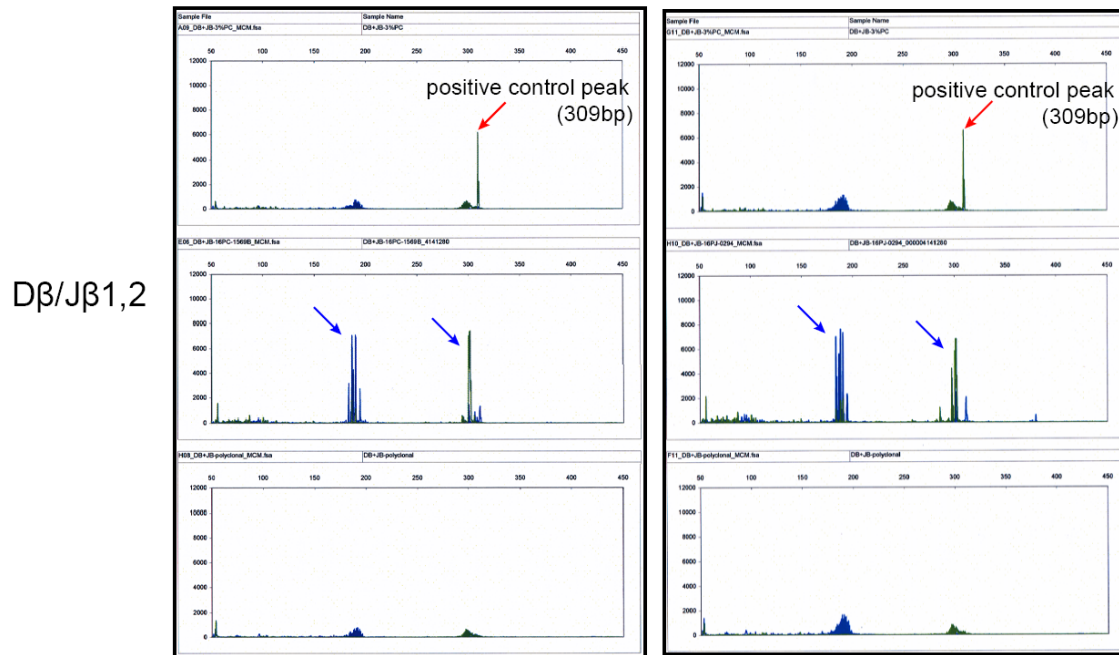
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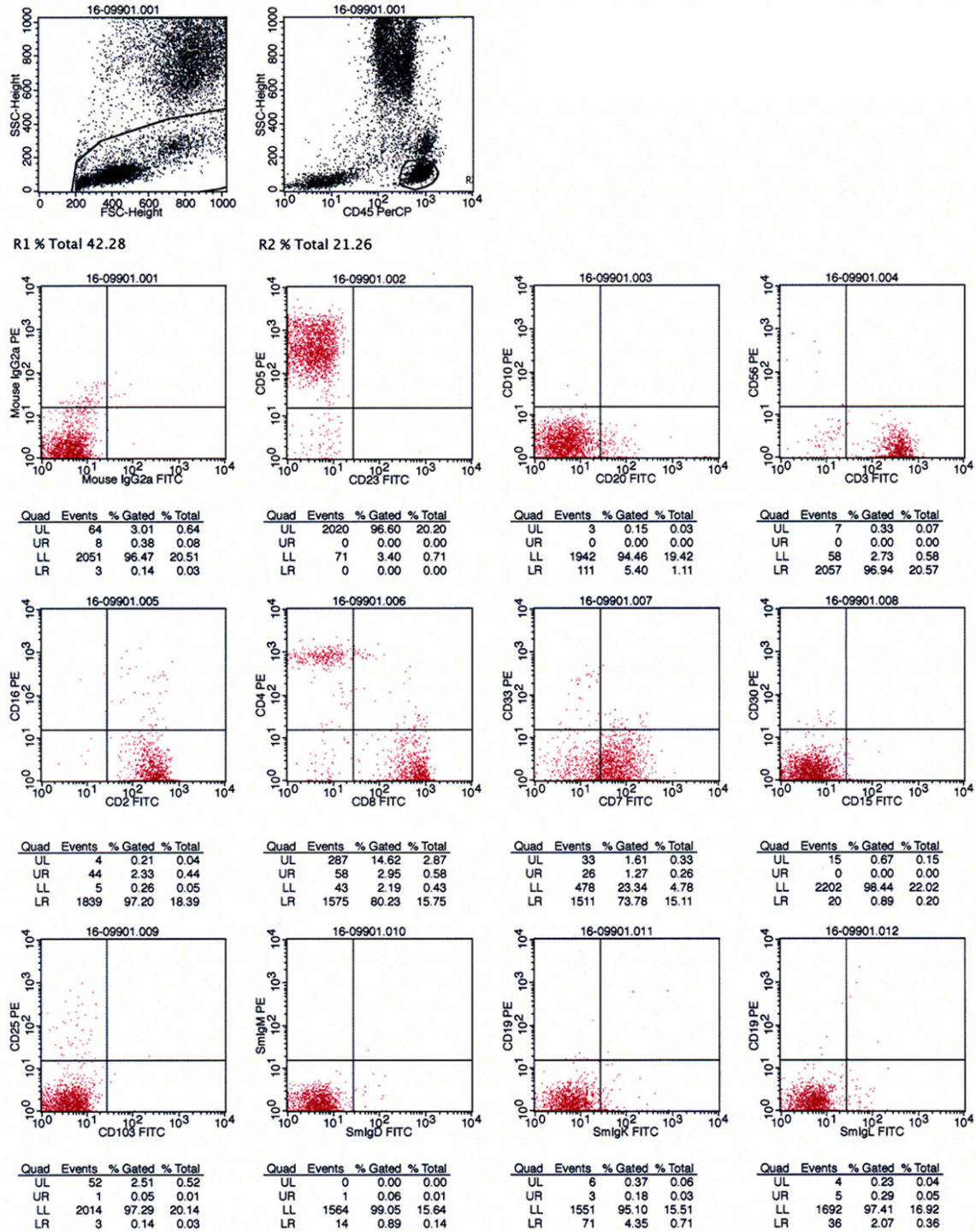


Nocardia meningitis in diffuse large B-cell lymphoma



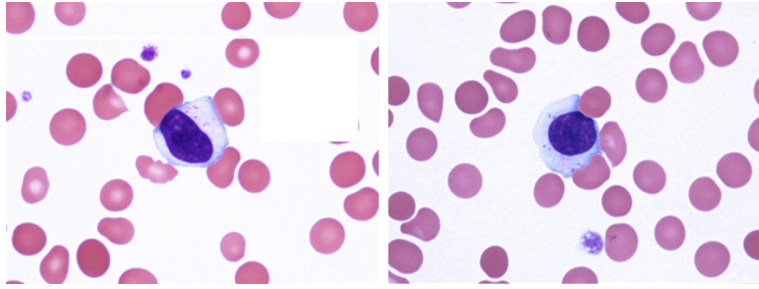
Supplementary Figure 1. Expression patterns of T-cell receptor (TCR) variable β (V β)/joining β (J β), and diversity β (D β) regions in the peripheral blood before and after chemotherapy. The clonality of the T-cells was identified by the comparison to a positive control peak level and to the peak's range. The clonality was identified as positive when the amplified peaks within the provisional range were higher than the control peak. TCR V β /J β 1, 2 showed a control peak (264 bp; red arrows) and oligoclonal green or blue peaks (blue arrows) in the provisional range from 240 to 285 bp. Blue peaks were from J β 2 and green peaks were from J β 1. TCR V β /J β 2 showed a control peak (253 bp; red arrows) and oligoclonal blue peaks in the provisional range from 240 to 285 bp. Blue peaks were from J β 2. TCR D β /J β showed a control peak (309 bp; red arrows) and oligoclonal green or blue peaks in two provisional ranges, from 170 to 210 bp and from 285 to 325 bp. Blue peaks were from J β 2 and green peaks were from J β 1.

Nocardia meningitis in diffuse large B-cell lymphoma



Supplementary Figure 2. Flow-cytometric analysis of the bone marrow cells. Very few CD20-positive cells were present (5.4%), but there were many CD3-positive cells (96%). The ratio of CD4-positive cells (14%) to CD8-positive cells (80%) was 0.18.

Nocardia meningitis in diffuse large B-cell lymphoma

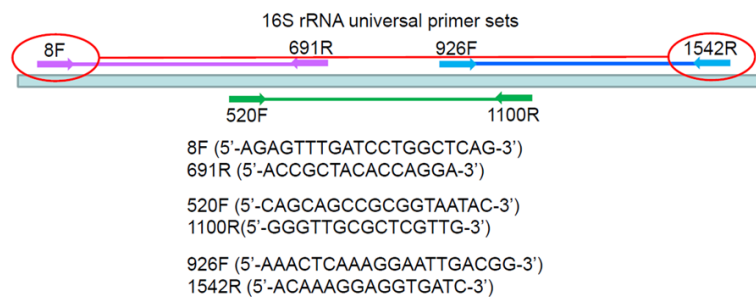


Supplementary Figure 3. Lymphocytes in the peripheral blood of the patient. The lymphocytes contained azurophilic granules in the cytoplasm (May-Giemsa stain at 400-fold magnification).

Supplementary Table 1. Susceptibility testing for Aerobic Mycobacteria, Nocardia, and other Actinomycetes; approved standard. This strain was cultured with Muller Hinton medium at 37 °C for 72 h

Antibiotics	Clinical and Laboratory Standards Institute M24-A			
	Susceptible	Intermediate	Resistant	This case
AMK	≤8	-	>16	1
ACV	<8/4	16/8	≥32/16	>32/16
CTRX	<8	16-32	≥64	>64
CPFX	≤1	2	≥4	4
IPM	≤4	8	≥16	>16
LZD	≤8	-	-	4
MINO	≤1	2-4	≥8	1
ST	≤38/2	-	≥76/4	19/1
TOB	≤4	8	≥16	2
CTX	≤8	16-32	≥64	>64
CFPM	≤8	16	≥32	>32
DOXY	≤1	2-4	≥8	1
GM	≤4	8	≥16	<0.5
ABPC				>8
CAM	≤2	4	≥8	>8
EM				>2

AMK: amikacin, ACV: potassium clavulanate, CTRX: ceftriaxone sodium, CPFX: ciprofloxacin hydrochloride, IPM: imipenem, LZD: linezolid, MINO: minocycline, ST: sulfamethoxazole/trimethoprim, TOB: tobramycin, CTX: cefotaxime, CFPM: cefepime dihydrochloride, DOXY: doxycycline hydrochloride, GM: gentamicin, ABPC: ampicillin, CAM: clarithromycin, and EM: erythromycin.



Supplementary Figure 4. 16S rRNA universal primer sets.

Nocardia meningitis in diffuse large B-cell lymphoma

