

Thymic function in MHC class II-deficient patients

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Background: MHC class II (MHC-II) molecules play a pivotal role in the development, activation, and homeostasis of CD4⁺ T_H cells in the thymus. The absence of MHC-II molecules causes severe T-cell immunodeficiency.

Objective: We sought to study thymic function, including T-cell receptor excision circle (TREC) quantification, in patients with MHC-II deficiency.

Methods: Eight MHC-II-deficient patients underwent a thorough T-cell immunologic work-up, including thymic activity, which was estimated based on TREC levels and T-cell receptor (TCR) genes, as well as analysis of several sequential human TCR gene rearrangements.

Results: *In vitro* responses to mitogens were normal or only slightly reduced, and flow cytometric evaluations of the TCR-V β repertoires of total CD3⁺ lymphocytes were normal in all patients. However, both the flow cytometric evaluation of the TCR-V β repertoire on CD4⁺ cells and spectratyping evaluation of the TCR-V γ repertoire on total CD3⁺ lymphocytes showed clonal abnormalities. TRECs were present in all patients in both total lymphocytes and sorted CD4⁺ cells. Additionally, TRECs were detected in genomic DNA obtained from Guthrie cards with dried blood spots. Quantitative RT-PCR assessment of different TCR gene rearrangement events revealed lower levels in MHC-II-deficient patients compared with levels seen in healthy control subjects. This was irrespective of the total lymphocyte numbers, suggesting a reduced global thymic activity.

Conclusions: Our report highlights potential pitfalls in diagnosing MHC-II deficiency and emphasizes the probable importance of MHC-II molecules in the normal thymic maturation process of T cells. Patients with MHC-II deficiency have detectable TRECs and might therefore be missed by a

TREC-based newborn screening program. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: MHC class II, immunodeficiency, severe combined immunodeficiency, T-cell receptor excision circle (TREC), T-cell receptor repertoire, neonatal screening

MHC class II (MHC-II) molecules play a pivotal role in the adaptive immune system because they direct the development, activation, and homeostasis of CD4⁺ T_H cells.¹ These molecules are also important for thymus development, and their lack of expression in the thymic epithelium is one of the reasons for impaired thymic maturation of CD4⁺ after hematopoietic stem cell transplantation (HSCT).² Hereditary defects involving 4 distinct regulatory proteins of MHC-II expression, defined as CIITA and subunits of RFX, have been recognized. These defects result in a severe T-cell autosomal recessive immunodeficiency disease called bare lymphocyte syndrome, which is also referred to as MHC-II deficiency.³ Inconsistent expression of MHC class I molecules and a lack of cellular and humoral immune responses to foreign antigens are additional characteristics of this disease. Affected patients are prone to severe and recurrent bacterial, viral, fungal, and protozoal infections beginning in the first year of life and resulting from inefficient CD4 T-cell development. Early diagnosis of patients with MHC-II deficiency is of great importance because prognosis is very poor, with many patients not surviving past a mean age of 4 years.^{4,5}

Diagnosis of other forms of T-cell immunodeficiencies is usually straightforward when patients present with the typical clinical features and a suggestive family history supported by the results of general immunologic tests. Reduced numbers of the different lymphocyte subsets in the peripheral blood and decreased responses of T cells to mitogen or antigen stimulation are typically seen in patients with a severe type of T-cell immunodeficiency, such as severe combined immunodeficiency (SCID). In some atypical cases an intensive work-up of the thymic activity, including analysis of the clonal pattern of the T-cell receptor (TCR) repertoires or enumeration of the T-cell receptor excision circles (TRECs), is required.⁶ The latter, which reflects thymic output, is even used as a neonatal screening assay for early diagnosis of severe T-cell lymphopenia.⁷

Although the mainstay of the diagnosis of MHC-II deficiency is the absence of constitutive and inducible expression of MHC-II molecules on all cell types, other tests are less informative. Direct activity of the thymus is rarely investigated in the evaluation of these patients. In this report we describe the clinical presentation, outcome, and relevant immunologic work-ups of 8 patients with MHC-II deficiency. Their actual thymic activity is presented, with a specific aim of gaining a better insight into the role of the MHC-II molecules in the developing thymocytes. This report highlights potential pitfalls in the diagnosis of MHC-II deficiency and

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Abbreviations used

Ct:	Cycle threshold
HSCT:	Hematopoietic stem cell transplantation
MHC-II:	MHC class II
qRT-PCR:	Quantitative real-time reverse transcriptase PCR
SCID:	Severe combined immunodeficiency
TCR:	T-cell receptor
TREC:	T-cell receptor excision circle

emphasizes the probable importance of MHC-II molecules in the normal maturation process of T cells in the thymus.

METHODS

Patients

All the patients in the Sheba Medical Center database who had a clinical phenotype of severe immunodeficiency together with the absence of HLA-DR expression suggestive of the diagnosis of MHC-II deficiency were identified, and their medical records were retrospectively evaluated. Approval for conducting this study was obtained from the Institutional Review Board of Sheba Medical Center, Tel Hashomer, Israel.

Immunologic work-up

PBMCs were isolated from freshly drawn heparin-treated blood by means of Ficoll-Hypaque density gradient centrifugation. Cell-surface markers, including the MHC-II molecule HLA-DR, were determined by means of immunofluorescent staining and flow cytometry (Epics V; Coulter Electronics, Hialeah, Fla) with antibodies purchased from Coulter Diagnostics. Lymphocyte proliferation in response to PHA and anti-CD3 stimuli was assessed based on titrated thymidine incorporation. Serum concentrations of immunoglobulins were measured by using nephelometry.

Quantitation of TCR genes

Representative samples of specific TCR-V β families on CD3⁺, CD4⁺CD8⁻, and CD4⁻CD8⁺ cells were detected and quantified by using flow cytometry (Coulter, Elite), as previously described.⁸ Anti-CD45RA (PE-CY7) and anti-CD45RO (Pacific blue) antibodies were used to detect the specific TCR-V β families on naive and memory/active T-cell subsets, respectively. TCR-V γ genes were amplified by means of PCR with fluorescence-labeled V γ primers, according to the standardized BIOMED-2 protocol.⁹ Fluorescence-labeled PCR products (1 μ L of each) were added to a mixture of 8.5 μ L of deionized formamide and 0.5 μ L of GeneScan 500 Rox internal lane standard (Applied Biosystems, Foster City, Calif) and separated with the 3100 Genetic Analyzer (Applied Biosystems). Results were analyzed with Gene Mapper software (Applied Biosystems). Profiles were characterized as oligoclonal if they contained 4 or fewer peaks, as polyclonal skewed if they had more than 4 peaks but were not Gaussian-like, and as polyclonal if they had more than 4 peaks in a Gaussian-like distribution.¹⁰

Quantification of TRECs

TREC levels were determined by using quantitative real-time reverse transcriptase PCR (qRT-PCR). Genomic DNA was extracted from patients' PBMCs or from patients' isolated CD4⁺ or CD8⁺ cells. qRT-PCRs were performed with 0.25 to 0.5 μ g of genomic DNA. Genomic DNA was also extracted from single 3.2-mm punches of dried blood disks (Guthrie papers) with the QIAamp DNAmicro kit (Qiagen, Hilden, Germany). DNA was eluted in 20 μ L of double distilled water, and 5 μ L was subjected to qRT-PCR. The PCRs contained TaqMan universal PCR master mix (Applied Biosystems), specific primers (900 nmol/L), and FAM-TAMRA probes (250 nmol/L), as previously described.¹¹ qRT-PCR was carried out in an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). The standard curve was

constructed by using serial dilutions of a known TREC plasmid (generously provided by Dr Daniel Douek, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, Md). The dilutions contained 10³ to 10⁶ copies of the signal joint TREC plasmid. A triplicate was used for each dilution. The obtained cycle threshold (Ct) values of the standard curve were the same in all experiments: 20.8 cycles for 10⁶ copies, 23.6 cycles for 10⁵ copies, 27.7 cycles for 10⁴ copies, and 30.1 cycles for 10³ copies. The number of TRECs in a given sample was automatically calculated by comparing the obtained Ct value of a patient's sample with the standard curve by using an absolute quantification algorithm. Amplification of RNaseP (TaqMan assay, Applied Biosystems) served as a quality control to verify similar amounts of genomic DNA that were used in the TREC analyses.

Isolation of CD4 and CD8 single-positive T cells

PBMCs were obtained by using Ficoll-Hypaque density gradient centrifugation. CD4⁺ or CD8⁺ T cells were isolated from PBMCs by means of positive selection with CD4 or CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, according to the manufacturer's instructions (98% purity). Quantitation of TCR genes and quantification of TRECs were performed as described above.

Analysis of human TCR gene rearrangement

The number of copies of various TCR gene rearrangements in a given sample was quantified by using qRT-PCR methodology, as described above. The primers and probes are listed in Table I. The obtained Ct was compared with the Ct of the TREC copies in the different standard serial dilutions, as described above, to calculate the TCR rearrangements for each patient. DNA samples obtained from 7 age-matched healthy subjects were used as controls. Each experiment was performed in triplicate, and the threshold line for Ct determination was positioned at the same level.

RESULTS

Clinical description and disease outcome

Eight patients were studied (Table II). A profoundly reduced HLA-DR expression was confirmed in 2 independent laboratories, suggesting the diagnosis of MHC-II deficiency in each of them. Seven patients had received diagnoses during infancy, and the eighth patient (patient 3) received a diagnosis at the age of 20 years. The latter patient has been reported in depth as a case of moderate combined immunodeficiency and long-duration clinical course.¹² The main reason for investigating this group of patients was severe infection. None had experienced any overt clinical autoimmune manifestations. Four patients underwent HSCT, and only 1 survived. Even the survivor required a second transplantation because his graft was lost 6 months after the first procedure. He is currently 1 year after a second transplantation and is alive and well, with 100% donor cells. Two patients died before HSCT was performed, and 1 patient is awaiting an HSCT from a match-related donor. The oldest patient (patient 3) is receiving intravenous immunoglobulin treatment and antibiotic prophylaxis and living a relatively normal life.

Immunologic work-up

All of our study patients had reduced CD4⁺ cell counts, ranging from 137 to 732 cells/mm³ (Table II). Five patients had a reverse CD4/CD8 ratio, and all patients had normal B-cell numbers. In 5 patients in whom enumeration of CD45RA on T cells was performed, results were variable, ranging from 42% to 93% of total CD3⁺ cells (normal range for age = 64% to 93% of total CD3⁺ cells). At the time of diagnosis, all 8 patients had reduced

TABLE I. List of primers used by qRT-PCR analysis to detect the amount of TRECs and the various TCR rearrangements

	Forward primer (5'-3')	Reverse primer (5'-3')	TaqMan probe (FAM-TAMRA, 5'-3')
TREC	CACATCCCTTTCAACCATGCT	GCCAGCTGCAGGGTTTAGG	ACACCTCTGGTTTTTGTAAAGGTGCCCACT
TCRD- δ /D2-D3	CAAGGAAAGGGAAAAAGGAAGAA	TTGCCCTGCAGTTTTTGTAC	ATACGCACAGTGCTACAAAACCTACAGAGACCT
TCRD- δ /D2-J1	AGCGGGTGGTGATGGCAAAGT	TTAGATGGAGGATGCCTTAACCTTA	CCCGTGTGACTGTGGAACCAAGTAAGTAAGTCTC
TCRD- δ /V1-J1	ATGCAAAAAGTGGTCGCTATT	TTAGATGGAGGATGCCTTAACCTTA	CCCGTGTGACTGTGGAACCAAGTAAGTAAGTCTC
TCRA-REC	AAAAAGCAACATCACTCTGTGTCT	GGCACATTAGAATCTCTCACTGA	CCAGAGGTGCGGGCCCCCA

TABLE II. Clinical and immunologic findings in 8 patients given a diagnosis of SCID: SCID MHC-II deficiency

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
HLADR ⁺ (in total lymph)	0%	0%	0%	0%	0%	0%	2%	0%
Age at diagnosis (mo)	6	6	240	3	6	4	3	4
Follow-up (mo)	3	24	180	3	3	30	10	1
Infections	+	+	+	+	+	+	+	+
Outcome	Died before HSCT	Well after second HSCT	Well on IVIG and PCP prophylaxis	Died 2 mo after HSCT, no engraftment	Died before HSCT	Died of CMV 24 mo after HSCT, no engraftment	Died of CMV and acute GVHD 3 mo after HSCT, no engraftment	Awaiting HSCT on IVIG and PCP prophylaxis
Lymphocyte count/mm ³	4900	3416	2148	1697	1872	5570	1890	7319
CD3 cells/mm ³	3552	1162	1890	390	1048	4450	930	3806
CD3 ⁺ CD4 ⁺ cells/mm ³	543	137	258	204	243	550	200	732
CD3 ⁺ CD8 ⁺ cells/mm ³	3305	109	1203	119	936	324	660	2708
CD4 ⁺ /CD8 ⁺ cells	0.16	1.25	0.21	1.71	0.25	1.69	0.30	0.27
CD19 ⁺ cells/mm ³	543	2186	170	71	300	730	780	2708
CD3 ⁺ CD45RA ⁺ cells	82%	85%	ND	93%	42%	ND	ND	79%
IgM (mg/dL)	UD	UD	25	UD	UD	UD	UD	UD
IgG (mg/dL)	253	UD	765 (on IVIG)	UD	927	UD	UD	UD
IgA (mg/dL)	UD	UD	6	UD	UD	UD	5	UD
IgE (mg/dL)	UD	UD	ND	UD	UD	UD	4	UD
PHA mitogenic response*	46.9%	94.8%	75%	106%	95%	116%	ND	106%
Anti-CD3 mitogenic response*	41.3%	32.8%	75%	150%	88%	170%	ND	40%
TREC levels/0.5 μ g of DNA†	674	1107	120	137	453	120	234	532
TREC levels/0.25 μ g of DNA CD4 ⁺	289	ND	82	ND	48	ND	ND	312
TREC levels/0.25 μ g of DNA CD8 ⁺	918	ND	76	ND	1042	ND	ND	1026

CMV, Cytomegalovirus; GVHD, graft-versus-host disease; IVIG, intravenous immunoglobulin; ND, not done; PCP, *Pneumocystis pneumonia*; UD, undetectable.

*Counts per minute patient/counts per minute control subject.

†The normal TREC level was greater than 828 copies per 0.5 μ g of DNA.

immunoglobulin levels, which were less than the level of detection in 5 of them. Lymphocyte activity, as determined based on cell response to various mitogen stimulations, was not significantly reduced and was even normal in 5 of the 7 subjects detected. Thus the rate of lymphocyte proliferation in the MHC-II-deficient patients ranged from 46.9% to 116% for PHA stimulation and 32.8% to 170% for anti-CD3 stimulation compared with healthy control values (Table II).

Thymic activity

TCR genes. Flow cytometric examination of the TCR-V β repertoire on total lymphocytes obtained from all the patients at presentation revealed normal polyclonal distributions (data not shown). Careful examination of the TCR-V β repertoire on CD4⁺ cells from 4 patients (Fig 1, A, patients 1, 3, 5, and 8), however, revealed some distribution abnormalities, including clonal expansion of the TCR-V β 7.1 (patients 1 and 5), TCR-V β 22 (patient 3), and TCR-V β 7.1, TCR-V β 8, and TCR-V β 13.2 (patient

8). In contrast, the TCR-V β repertoire on CD8⁺ cells from these 4 patients (Fig 1, B) revealed normal distribution, excluding clonotype expansion within the CD8 cells. Analysis of the TCR-V β repertoire in total lymphocytes separately on CD4⁺ and CD8⁺ subsets and within naive (CD45RA⁺) and memory/active (CD45RO⁺) subsets for each of them was performed in 2 patients (patients 5 and 8, Fig 2). This analysis revealed clonal expansion of mainly CD4⁺CD45RA⁺ cells (TCR-V β 7.1, TCR-V β 8, and TCR-V β 21.3 and TCR-V β 7.1 in patients 5 [Fig 2, A] and 8 [Fig 2, B], respectively) compared with CD4⁺CD45RO⁺ cells (only TCR-V β 8 in patient 8; Fig 2, D). Total lymphocytes from all patients were examined by spectratyping for the TCR-V γ repertoires to better define T-cell clonality (Fig 3). Although it is not characteristically expressed on the cell surface, the γ chain gene remained rearranged in the T-cell genome and provided a conventional marker of clonality because of its presence in both the TCR α β and TCR γ δ T cells. Four different TCR-V γ rearrangement events were analyzed in each patient. All 4 were abnormal (ie, either oligoclonal, skewed polyclonal, or undetectable) in 2

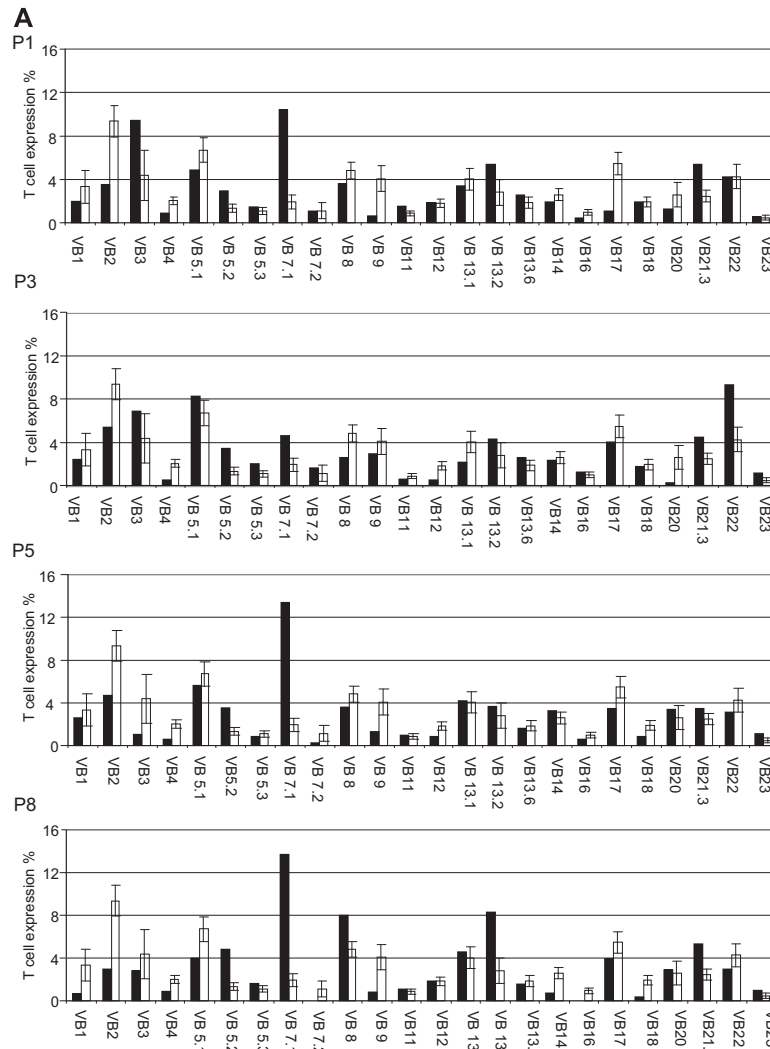


FIG 1. TCR repertoire: FACS analyses on CD3⁺CD4/CD8⁺ cells. Relative expression of 24 different TCR-Vβ families in CD3⁺CD4⁺ cells (A) and CD3⁺CD8⁺ cells (B; solid bars) obtained from 4 MHC-II-deficient patients' PBMCs compared with the relative expression of normal CD3⁺CD4⁺ or CD3⁺CD8⁺ control cells (open bars). Normal control values were obtained from the IOTest Beta Mark-Quick Reference Card (Beckman Coulter, Fullerton, Calif). P, Patient.

patients (patients 3 and 4). Two rearrangements were abnormal (skewed polyclonal) in 1 patient (patient 8), and 1 rearrangement was abnormal (skewed polyclonal) in 5 patients (patients 1, 2, and 5-7). Interestingly, the same TCR-Vγ (VγF1) underwent abnormal rearrangement in all patients (Fig 2).

Quantification of TRECs. TREC levels on total lymphocytes were studied in all patients at the time of diagnosis and were within detectable levels (range, 120-1107 copies per 0.5 μg of DNA) compared with levels seen in 19 healthy infants (>828 copies per 0.5 μg of DNA, Table II). There was no correlation between those TREC levels and clinical outcome. TREC levels in 4 patients (patients 1, 3, 5, and 8) were analyzed on sorted CD4⁺ and CD8⁺ cells (Table II). All 4 patients had detectable TREC levels in both CD4⁺ and CD8⁺ sorted cells. TREC levels on CD4⁺ cells were significantly lower than on CD8⁺ cells in 3 patients, possibly reflecting a reverse ratio of CD4/CD8 cells and abnormal CD4 development. In patient 3 TREC levels on CD4⁺ and CD8⁺ cells were similar, despite the reversed CD4/CD8 ratio in

peripheral blood. However, her overall levels were low, possibly reflecting peripheral dilution of TRECs caused by her relatively older age (Table II). We performed TREC analysis on genomic DNA obtained from Guthrie cards with dried blood spots from 2 patients. Similar to peripheral blood, the DNA from the Guthrie cards of patients 2 and 8 showed a detectable number of TRECs (113 and 100 copies per 3-mm punched disk, respectively; Fig 4). These results were similar to TREC levels from genomic DNA obtained from a Guthrie card with dried blood spots of 15 healthy newborns (35-212 copies per 3-mm punched disk) and in marked contrast to 7 patients with confirmed SCID in whom TREC copies were undetectable or significantly low (Fig 4).

Analysis of different TCR gene rearrangements. The TCR is created through a sequential order of recombination events between the different TCR genes (TCRδ > TCRγ > TCRβ > TCRα). A sequential order also occurs within each particular gene. For example, the Dδ2 to Dδ3 rearrangement occurs earlier than the Dδ2 to Jδ1 rearrangement

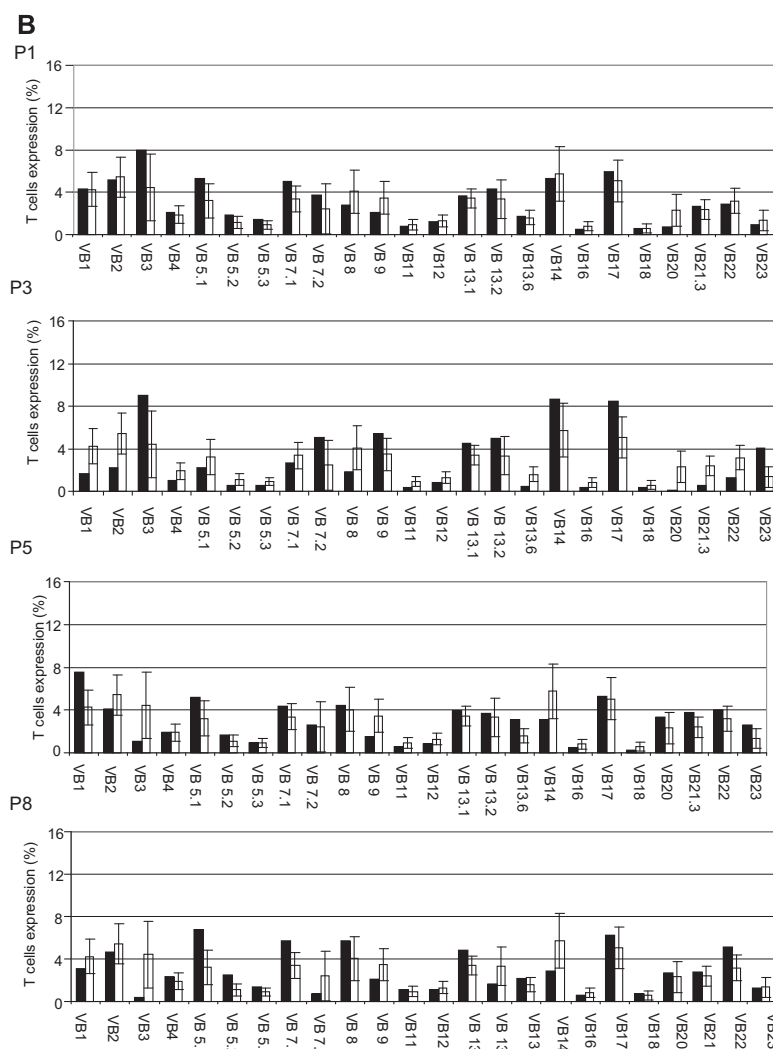


FIG 1. (Continued)

in TCRD, and the V δ to J δ 1 rearrangement occurs even later. Thus different DNA segments are excised during this sequential process.¹³ To better test thymocyte maturation in patients with MHC-II deficiency, we measured 4 different TCR gene rearrangement events that represent different stages of thymocyte development within the thymus (Fig 5, A). Cells obtained from 5 affected infants were analyzed by using qRT-PCR (patients 1, 2, 4, 5, and 8). The average quantity of the TCR δ -D δ 2-D δ 3 rearrangement in all 5 patients was similar to that seen in healthy infant control subjects (139 ± 38.2 vs 187 ± 98 copies, respectively). However, the averages of the quantities of the remaining TCR δ -D δ 2-J δ 1, TCR δ -V δ 1-J δ 1, and TCR α -REC rearrangements were markedly reduced compared with those in healthy control subjects (95 ± 46.4 vs 377 ± 217.1 copies, 13 ± 7 vs 54 ± 23.8 copies, and 358 ± 201.3 vs 1858 ± 608.2 copies, respectively; Fig 5, B).

DISCUSSION

Patients with MHC-II deficiency typically present with infections suggestive of significant T-cell immunodeficiency early in life.⁴ Although their total numbers of circulating T and B lymphocytes might be normal, the ratio of CD4⁺ to CD8⁺ T cells is

reversed in most of them because of the reduction in CD4⁺ cell counts. The lack of MHC-II expression on cortical thymic epithelial cells is also expected to lead to reduced thymic activity. Yet in mice MHC-II molecules appear as focal aggregates on the surface of cortical thymic epithelial cells, but their formation is independent of TCR or TCR-bearing cells.^{14,15} Indeed, although reduced in numbers, the CD4⁺ T cells in patients with MHC-II deficiency do not exhibit major phenotypic abnormalities, and only relatively minor differences in the TCR repertoire of CD4⁺ T cells have been described in these subjects.¹⁶ Our results demonstrated that evaluation of TCR repertoires in total lymphocytes from patients with MHC-II, as determined by using flow cytometric analysis, was normal. Therefore we suggest that assessing the variability of the separated cell populations is a more sensitive way to detect TCR-V β clonal abnormalities. Indeed, specific evaluation of the TCR repertoires in CD4⁺ cells showed abnormal clonal expansions, whereas the expansion in the CD8⁺ population was normal. Moreover, the clonal expansion was more dominant in CD4⁺ naive cells compared with that seen in CD4⁺ memory/active cells. In addition, spectratyping examination of the TCR-V γ gene clonality, which represents the “prototype” of restricted repertoire targets, revealed abnormal patterns in nearly one half of the

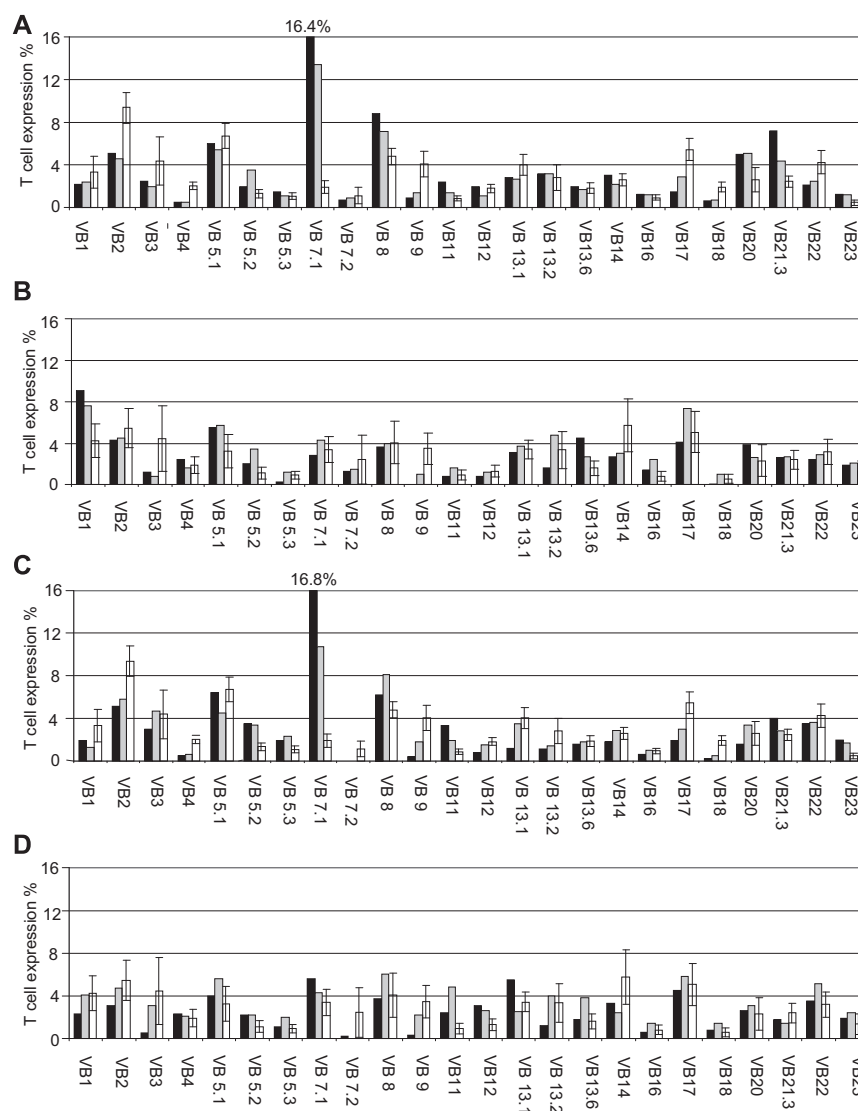


FIG 2. TCR repertoire: FACS analyses on $CD3^+CD4/CD8^+CD45RO^+/CD45RA^+$ cells. Relative expression of 24 different TCR-V β families in $CD3^+CD4/CD8^+CD45RA^+$ (solid bars) and $CD3^+CD4/CD8^+CD45RO^+$ (shaded bars) cells obtained from 2 MHC-II-deficient patients' PBMCs compared with the relative expression of normal $CD3^+CD4^+$ or $CD3^+CD8^+$ control cells (open bars). Normal control values were obtained from the IOTest Beta Mark-Quick Reference Card (Beckman Coulter). **A**, CD4 cells from patient 5. **B**, CD8 cells from patient 5. **C**, CD4 cells from patient 8. **D**, CD8 cells from patient 8.

examined rearrangements. This abnormality can suggest a reduced N region or low exonuclease activity, resulting in the formation of a clonal pattern. This is in agreement with the findings of Henwood et al,¹⁷ who detected no significant differences when the lengths of complementarity-determining region 3 or the amount of nongermline modifications at the sites of recombination were compared between patients and control subjects. However, a more detailed analysis of the specific TCR-V β 6 rearrangements that had been derived from the $CD4^+CD8^-$ subsets from their patients revealed abnormal patterns. This observation by Henwood et al¹⁷ is similar to ours, and it suggests that MHC-II molecules are important for the formation of normal antigen-binding sites and that their absence results in property alterations.

As previously observed by Canoni et al,⁵ a routine T-cell work-up of patients with MHC-II deficiency is not informative.

Although almost all of our patients displayed a reverse $CD4/CD8$ ratio, the number of $CD4^+$ cells was not completely absent. Even enumeration of $CD45RA$, representing naive T cells, was inconclusive. Moreover, all patients had near-normal or even normal lymphocyte functions, as determined by their responses to various mitogen stimulations. At the same time, determination of different DNA rearrangements in 5 of these 8 patients revealed reduced numbers in most of the tested rearrangements. On the basis of the known order of these rearrangements, which correlates with the T-cell maturation stages inside the thymus,¹³ no specific developmental block could be determined. These results are indicative of a nonspecific thymic dysfunction in patients with MHC-II deficiency.

Detection of TRECs on Guthrie cards with dried blood spots was recently suggested as being a sensitive neonatal screening test capable of identifying SCID and other T-cell lymphopenias.^{18,19}

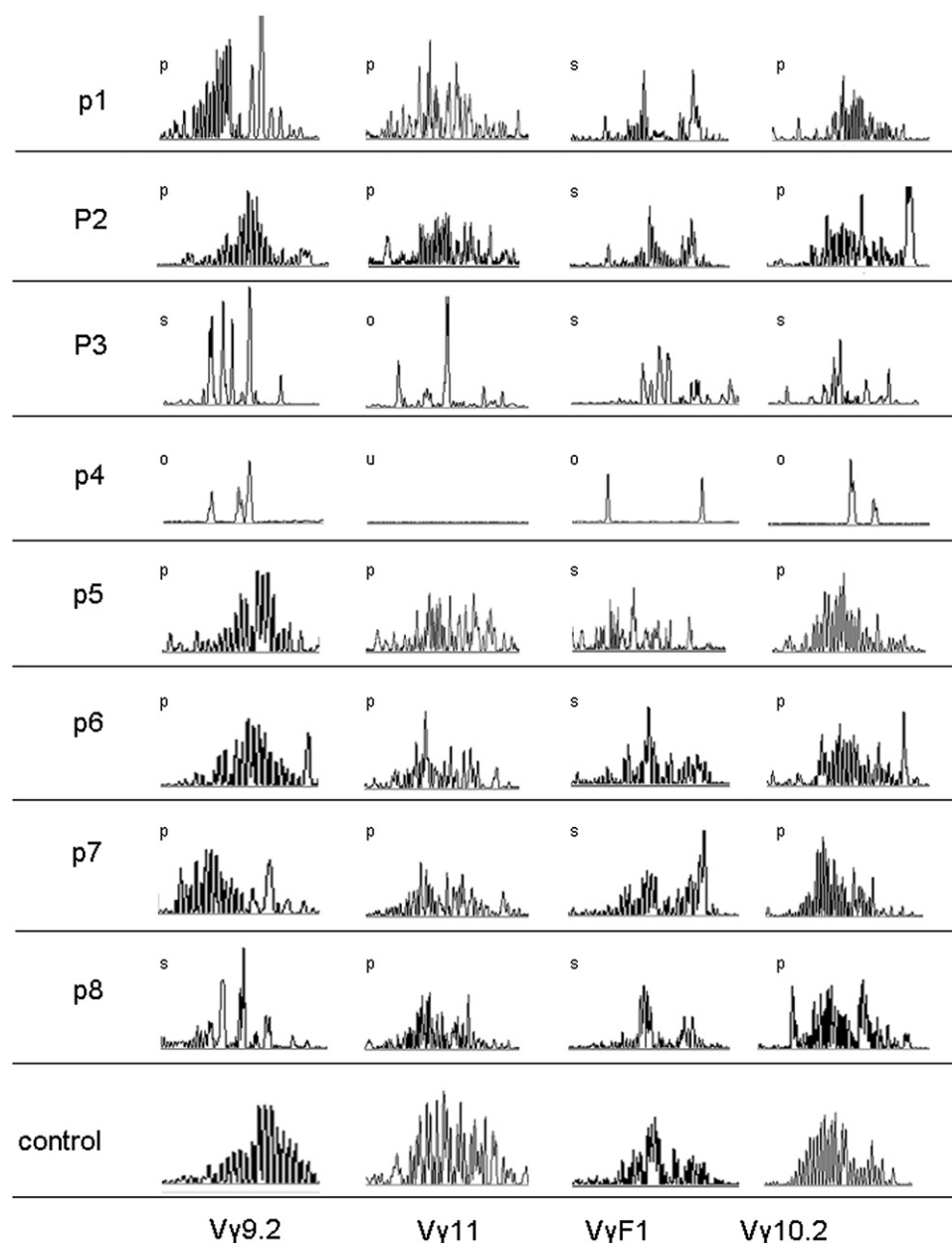


FIG 3. Rearrangement analysis of TCR-V γ in MHC-II-deficient patients' PBMCs. Four different V γ rearranged TCR genes (V γ 9.2, V γ 11, V γ F1, and V γ 10.2) were PCR amplified in 8 MHC-II-deficient patients and age-matched healthy control subjects, followed by Gene Scan analysis. Four different patterns are shown: p, polyclonal; s, skewed polyclonal; o, oligoclonal; u, undetected.

Moreover, a recent report showed that the TREC assay is also able to diagnose significant cellular immune defects, even though such patients have residual T lymphocytes.²⁰ No false-negative results have thus far been reported in pilot studies on the use of such assays.²¹ Notably, all of our MHC-II-deficient patients had detectable TREC levels. Moreover, even their CD4⁺CD8⁺ subsets had detectable TREC levels. Therefore such patients could be missed in neonatal screening by using Guthrie cards. We did find a correlation between the findings from the Guthrie cards and the TREC levels in the peripheral blood of our 2 patients who underwent TREC analysis on Guthrie cards. We can assume there would have been similar correlations in the other MHC-II-deficient patients. Therefore physicians who encounter patients presenting

with clinical pictures that raise suspicion of immunodeficiency and who exhibit normal TREC newborn screen results or normal peripheral blood TREC levels on diagnosis still need to perform thorough immune evaluations, at least to exclude MHC-II deficiency. Thus MHC-II deficiency is a disorder that is an example of a possible pitfall when we use TRECs for screening.

Similarly, we speculate that other diseases with residual T cells, partially functional T cells, or both with numerous CD45RA⁺ cells and relatively normal thymic architecture and function might be missed by a TREC-based newborn screening assay. This has recently been suggested by Roifman et al²² and Buckley.²¹ Such diseases include ζ chain-associated protein of 70 kDa (ZAP70) deficiency, a specific R222C common

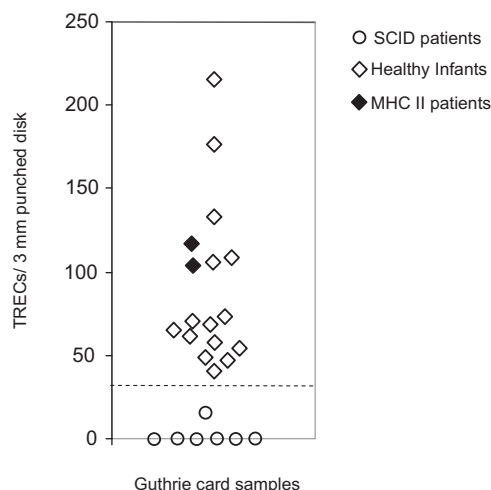


FIG 4. TREC copy numbers in dried blood spots. TRECs were determined on Guthrie cards with dried blood spots obtained from 15 anonymized healthy infants (open diamonds), 7 patients with SCID (open circles), and 2 MHC-II-deficient patients (solid diamonds). The dashed line represents cut-off values for TREC levels per 3-mm punched disk.

γ chain mutation, CD3 γ deficiency, CD8 deficiency, or patients with well-defined combined immunodeficiencies, such as ataxia telangiectasia, hyper-IgM syndrome, Wiskott-Aldrich syndrome, most cases of DiGeorge syndrome, and IL-10 receptor deficiency.

The outcome of patients with MHC-II deficiency was reported as being poor, even after HSCT.²³ This was also the case in our study, in which 3 of our 4 patients who underwent HSCT did not have engraftment and succumbed to infections. Only 1 patient survived HSCT, but he required a second transplantation. The main causes of treatment failure in patients with MHC-II deficiency is poor engraftment because of residual host immunity, old age on diagnosis, and/or treatment and detrimental influence of infections. Matheux and Villard¹⁶ reported that engraftment had not been adequately achieved, even in an uninfected infant given a diagnosis immediately after birth who received additional immunosuppressive conditioning to destroy competent host T cells before HSCT. Recently, a better outcome was reported, especially in HLA-identical transplant recipients, in which the overall disease-free survival rate was 66% after a median follow-up of 6.3 years.²⁴ In addition, Ouederni et al²⁵ reported that 10 of 23 patients who underwent HSCT were cured, with the recovery of almost normal immune function. Seven patients in their study who did not undergo HSCT are still alive and receiving immunoglobulin treatment and antibiotic prophylaxis, indicating that some patients can survive for relatively long periods providing that multiple prophylactic measures are implemented, as in the case of our older patient (patient 3).

In summary, we showed here that patients with MHC-II deficiency have reductions in some thymic activities, indicating the role of MHC-II molecules not only in thymic selection of CD4⁺ thymocytes but also throughout the development of thymocytes and the TCR rearrangement process in the thymus. In addition, CD4⁺ cells, rather than total lymphocytes, should be evaluated to further estimate clonal expansion of a patient's lymphocytes. TREC quantification, which is successfully used as a neonatal screening assay in the diagnosis of patients

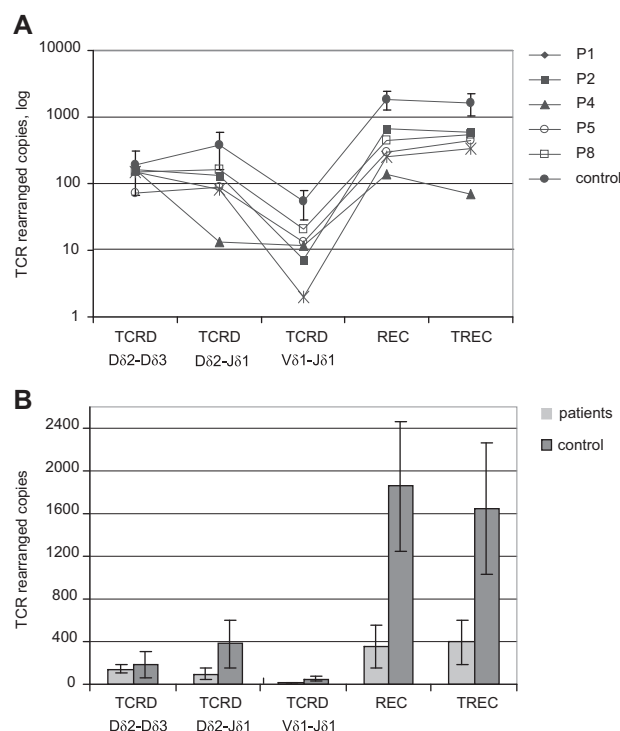


FIG 5. Analysis of the ordered TCR rearrangement events in MHC-II-deficient patients' PBMCs. Five different TCR rearrangement events (TCRD-D δ 2-D δ 3, TCRD-D δ 2-J δ 1, TCRD-V δ 1-J δ 1, TCRA-REC, and TREC) were analyzed by using qRT-PCR in PBMCs obtained from 5 MHC-II-deficient patients and 7 age-matched healthy control subjects. The control is presented as the average $\pm$ SD of the 7 samples. For each patient, the TCR rearrangements are presented as DNA copies in log scale. The obtained Ct in each detected point was compared with the Ct of the TREC copies in the different standard serial dilutions, as described in the text (A). For each TCR rearrangement, the averages of the results obtained either for the 5 patients or for the 7 control subjects were calculated and compared. SDs of the calculated averages are included (B).

with significant T-cell immunodeficiency, might not detect this group of patients. Therefore we suggest that patients with MHC-II deficiency are the first significant T-cell immunodeficiency group of patients who could be missed (false-negative result) by TREC analysis in their DNA obtained from either peripheral blood or Guthrie cards with dried blood spots.

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Clinical implications: Neither normal TREC screening results nor detectable TREC levels on clinical diagnosis can independently exclude suspected MHC-II deficiency.

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