Brief Definitive Report

T Cell Receptor Repertoire in Polymyositis: Clonal Expansion of Autoaggressive CD8+ T Cells

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Summary

In polymyositis (PM), CD8+ T cell receptor (TCR) α/β^+ cells invade and destroy major histocompatibility complex class I–positive muscle fibers. We combined polymerase chain reaction (PCR) and double-fluorescence immunocytochemistry to analyze the T cell receptor (TCR) repertoire expressed in muscle of PM patients. In patient 1, inverse PCR revealed a preferential usage of TCR Vα33.1, Vβ13.1, and Vβ5.1. Six of six TCR Vα33.1+ clones and five of seven Vβ13.1+ clones had identical nucleotide sequences. In contrast, the Vβ5.1+ TCRs were more heterogeneous. Similar results were obtained with an independent PCR method using primers specific for TCR Vα33, Vβ13, or Vβ5. No TCR sequences could be amplified from noninflammatory control muscle. Furthermore, none of the TCR sequences found in PM muscle could be detected in blood from the same patient or from a normal control subject. Immunohistochemistry confirmed that Vβ5.1 and Vβ13.1 were overrepresented in the muscle lesions of this patient. 32% of all CD8+ T cells were Vβ13.1+, and 16% were Vβ5.1+. However, ~60% of the CD8+ T cells that invaded muscle fibers were Vβ13.1+, whereas 10% were Vβ5.1+. In patient 2, 50% of the T cells were Vβ5.1+, and as in patient 1, these T cells were mainly located in interstitial areas. In patient 3, >75% of the autoinvasive T cells stained with an anti-Vβ3 mAb. Sequence analysis of 15 PCR clones amplified with a Vβ3-specific primer showed that 9 (60%) sequences were identical. The results suggest that (*a*) a strikingly limited TCR repertoire is expressed in PM muscle; (*b*) there is a dissociation between the TCR usage of autoinvasive and interstitial T cells; and (*c*) the autoinvasive T cells are clonally expanded.

Polymyositis (PM) is presumably an autoimmune disease caused by T lymphocytes that surround, invade, and destroy HLA class I–expressing muscle fibers (1–3). With few exceptions (4, 5), the muscle-infiltrating T lymphocytes are CD8+ α/β T cells. Attempts to identify viral target antigens or related gene sequences in muscle fibers have failed, and it is generally considered that the recognized epitope is probably a muscle-derived auto-Ag (1–3, 6).

The characteristic lesion of PM has several unique features that make it an ideal paradigm to study CD8+ T cell–mediated immunopathology. The target cells, multinucleated skeletal muscle fibers, can be readily distinguished from the effector T cells. A subpopulation of CD8+ T cells invade muscle fibers. These "autoinvasive" T cells are presumably enriched in auto-Ag–specific cells (1–3). Here we have applied immunohistochemistry and PCR techniques to characterize the TCR repertoire of inflammatory cells in PM muscle.

Materials and Methods

Clinical Material. Muscle biopsy specimens and PBMC were obtained from patients with typical PM (1–3). HLA types are known for patient 1 (HLA A2/32, B27/44, Cw2/w3, DR2/w11) and patient 2 (A26/32, B8/51, $C^{-/-}$, DR3/w10). Control muscle tissue was obtained during diagnostic biopsy from a patient with noninflammatory myopathy.

Immunohistology. 10-μm cryostat sections of muscle were used for paired immunofluorescence studies with FITC–anti-CD8 (10 μg/ml; Dianova, Hamburg, Germany) and the following mouse anti-TCR mAbs: Vβ5.1, 5.2, 5.3, 6.7, 8, and 12.1 (diluted 1:10; T Cell Sciences, Cambridge, MA); Vβ2, 3, 13.3, 16, 17, 18, 21.3, and 22 (diluted 1:10; Immunotech, Marseille, France); Vβ7.1, 13.1, and 13.2 (undiluted supernatants; kindly provided by Dr. M. Callan, Molecular Immunology Group, John Radcliffe Hospital, Oxford, UK [Vβ7.1] and Dr. P. Marrack, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO [7]). The TCR-specific primary antibodies were immunolocalized with carboxymethylindocyanine (Cy3, 10 μg/ml; Dianova)-labeled goat anti-mouse IgG. Isotype-matched IgG at identical concentrations was substituted for primary antibodies for negative controls.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from frozen muscle and freshly isolated PBMC with the guanidinium thiocyanate method (8). Single-stranded cDNA was synthesized from 2 μg of total RNA using oligo(dT) and Moloney murine leukemia virus–derived reverse transcriptase (GIBCO/BRL, Eggenstein, Germany).

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Family PCR. For family PCR, single-stranded cDNA was used as template. TCR α and β chains were amplified with 5′-V gene family–specific primers (Vα15: 5′-GGAATTCCAAGACCAAAG-ACTCACTG-3′; Vα33: 5′-GGAATTCGCAGCTTCTTCAGA-GAGAG-3′; Vβ3: 5′-GGAATTCTAGAGAGAAGAAGGAGCGC-3′; Vβ5: 5′-GGAATTCTCCTGGTACCAACAG (GA) CCC-3′; Vβ13: 5′-GGAATTCCCC (CT) (GA) ATGGCTACAATGTC-3′) and 3′-primers for the Cα and Cβ regions (Hcα6: 5′-CAATGG-ATCCTTGTCACTGGATTTAGAGTC-3′ or Hcβ2: 5′-GGGTCG-ACGGTGTGGGAGATCTCTGC-3′).

Inverse PCR. For inverse PCR (9), double-stranded cDNA was generated using RNAse H, *Escherichia coli* DNA polymerase I, and *E. coli* DNA ligase, followed by incubation with T4 DNA polymerase for blunt-end formation. The blunt-ended cDNA was circularized with T4 DNA ligase (GIBCO/BRL) and used as template. Two primer pairs, each containing a forward and reverse TCR C region sequence, were used to amplify TCR α chains (HcαiII: 5′-GGAATTCCTGCTATGCTGTGTGTCTGG-3′; Hcα6: see above) and TCRβ chains (HcβiIII: 5′-GGAATTCTGTCTGCCACCATC-CTCTATGAG-3′; Hcβ2: see above). All primers contained artificial EcoRI (5′ primers) or BamHI or BglII (3′ primers) restriction sites. Each reaction was carried out in a total volume of 50 μl containing 1 U *Taq* DNA polymerase (Angewandte Gentechnologie Systeme GmbH [AGS], Heidelberg, Germany), 200 μM of each deoxynucleotide triphosphate, 150 μM $MgCl_2$, and 50 pmol of each primer in buffer supplied by the manufacturer (AGS). The PCR was done for 35 cycles with 1 min denaturation at 94°C, 1 min annealing at 60°C, 1 min extension at 72°C, and a final extension for 10 min at 72°C in a thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT).

Cloning and Sequencing of PCR Products. PCR products were cloned into M13mp18 for sequence analysis. TCR Vα samples were digested with BamHI and EcoRI and TCR Vβ samples with BglII and EcoRI to cut restriction sites in the oligonucleotide primers. DNA fragments were separated by electrophoresis on a 1% low melting point agarose gel and fragments of expected size were isolated by jet sorb (Genomed, Bad Oeynhausen, Germany). The isolated DNA fragments were cloned into BamHI- and EcoRI-digested M13mp18. After transfection into the *E. coli* strain DH5αFI^q (GIBCO BRL), clones containing TCR Vα or Vβ inserts were identified by hybridization with TCR Cα– or Cβ–specific oligos (xHcα: 5′-CACGGCAGGGTCAGGGTTCT-3′ or xHcβ3: 5′-GAA-CAC (GC) TT (GT) TTCAGGTCCT-3′). Positive clones were sequenced by using the dideoxy chain termination method (10) with the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH). The cDNA sequences were screened against Genbank and EMBL nucleotide sequence databases. The TCR nomenclature is used according to references 9, 11–16. Some Vα and Vβ elements were reclassified: Vα29.1 and Vα33.1 were previously designated Vαw23 and Vα31, and members of the Vβ13 family were previously designated Vβ12.3 and Vβ12.4.

Results and Discussion

In a first stage of analysis, we studied the TCR α and β chain repertoire expressed in the muscle of patient 1 by inverse PCR. In contrast to TCR "family PCR," inverse PCR is independent of V gene–specific primers, allowing the unbiased identification of the expressed TCR V gene repertoire, including hitherto unknown V genes (9).

While no TCR sequences could be amplified from noninflammatory control muscle, 33 Vα and 32 Vβ clones were amplified and sequenced from PM muscle. In parallel, 22 Vα and 28 Vβ clones were amplified and sequenced from the PBMC of a healthy subject.

We found 17 different Vα and 17 Vβ segments in muscle. Two Vβ elements (Vβ5.1, 7/32 [22%] clones; Vβ13.1, 7/32 [22%]) and four Vα elements (Vα7, 5/33 clones [15%]; Vα8.1, 4/33 [12%]; Vα15.1, 3/33 [9%]; Vα33.1, 6/33 [18%]) were clearly enriched. The TCR Vα and Vβ repertoire of PBMC from the healthy donor was broader. Vβ5.1 and Vβ13.1 were expressed by 3/28 (10%) and 2/28 (7%) of the PBMC clones, respectively. No Vα33.1$^+$ clone was found in PBMC. All Vα and Vβ sequences amplified from PBMC were different (not shown).

Sequence analysis of the TCR Vβ clones derived from PM muscle indicated that there was striking clonal expansion of Vβ13.1$^+$ T cells. Five of seven Vβ13.1$^+$ clones had identical sequences (Table 1 *B*). In contrast, the Vβ5.1 sequences, which were expressed as frequently as Vβ13.1, were more heterogeneous (Table 1 *B*).

Analysis of the Vα chains expressed in muscle also produced evidence for clonal expansion. Six of six Vα33.1$^+$ clones, three of three Vα15$^+$ clones, and three of five Vα7$^+$ clones were identical (Table 1 *A*). In contrast, all four Vα8.1$^+$ clones had different CDR3 sequences (not shown). All TCR β chains and 31 of the 33 α chains were productively rearranged.

The results obtained with inverse PCR were confirmed with "family PCR," using primers specific for Vα33, Vβ5, and Vβ13. Seven of eight Vα33$^+$ clones and five of eight Vβ13$^+$ family PCR–derived clones had the same sequence as six of six Vα33$^+$ and five of eight Vβ13$^+$ inverse PCR–derived clones (Table 1, *A* and *B, underlined sequences*). In contrast, only two of the nine family PCR–derived Vβ5$^+$ clones were identical to one of nine Vβ5$^+$ clones derived by inverse PCR (Table 1 *B*). All Vβ13.1$^+$ and one of the Vβ5.1$^+$ sequences that shared identical CDR3 regions were rearranged to Jβ1.5 and Cβ1 (Table 1 *B*).

We also analyzed PBMC from the same PM patient for TCR Vα33, Vβ5, and Vβ13 expression, using TCR family PCR. None of the TCR sequences found in the muscle could be detected in the blood (not shown). Furthermore, by FACScan® analysis (Becton Dickinson Immunocytometry Systems, Mountain View, CA), 2% of the CD4$^+$ cells and 1.3% of the CD8$^+$ cells were Vβ13.1$^+$; 2.8% of the CD4$^+$ cells and 0.9% of the CD8$^+$ cells were Vβ5.1$^+$. The total proportion of CD4$^+$ cells in the blood was 57.5%, and the proportion of CD8$^+$ cells was 28.9%. These results are consistent with the assumption that Vα33.1$^+$, Vβ5.1$^+$, and Vβ13.1$^+$ T cells accumulated or selectively expanded in the muscle. However, the blood sample was obtained later than the muscle biopsy after treatment with immunosuppressive drugs. Therefore, the possibility that the difference in TCR expression between blood and muscle was related to therapy cannot be excluded.

It is tempting to directly relate the clonal expansion of T cells to the pathogenesis (reviewed in reference 17). However, clonal populations of CD8$^+$ T cells were observed in the blood of normal elderly human subjects and mice (18–20). As pointed out by Posnett et al. (18), the presence of en-

Table 1. *Sequence Analysis of TCR Expression in PM Lesions*

(A) TCR Vα sequences (patient 1)

Vα33	N	Jα	f*
Inverse PCR			
33.1	LGGSGTY‡	17.10	6/6
Family PCR			
33.1	LGGSGTY‡	17.10	7/8
33.1	LGRETSYD	13.3	1/8

(B) TCR Vβ sequences (patient 1)

Vβ5	N-Dβ-N	Jβ	Cβ	f*	Vβ13	N-Dβ-N	Jβ	Cβ	f*
Inverse PCR					Inverse PCR				
5.1	IGQGM	1.1	1	2/9	13.1	GVRGGY‡	1.5	1	5/8
5.1	GSGG	1.5	1	2/9	13.1	YSGQGNP	1.1	1	1/8
5.1	LAGQP	1.1	1	1/9	13.1	YSPQGW	1.2	1	1/8
5.1	RGQGFG	1.6	1	1/9	13.4	LQGSPR	1.1	1	1/8
5.1	SGQGPPHG‡	2.7	2	1/9					
5.2	LGNSGWV	1.1	1	1/9					
5.3	EGL	1.2	1	1/9					
Family PCR					Family PCR				
5.1	RGQGGG	2.5	2	2/9	13.1	GVRGGY‡	1.5	1	5/8
5.1	SGQGPPHG‡	2.7	2	2/9	13.1	RPDRGRA	1.4	1	1/8
5.1	LGQGPE	1.2	1	1/9	13.1	YEAGPD	2.7	2	1/8
5.1	NGQGL	1.2	1	1/9	13.2	YSGAESW	1.5	1	1/8
5.1	LGDRGQD	1.2	1	1/9					
5.1	SWTGEGTDT	2.7	2	1/9					
5.3	GLAGGP	2.1	2	1/9					

(C) TCR Vβ sequences (patient 3)

Vβ3	N-Dβ-N	Jβ	Cβ	f*
Family PCR				
3	FRGGVH	2.7	2	9/15
3	LLASGSLGY	2.7	2	2/15
3	LRTDA	1.4	1	2/15
3	TRVGV	1.1	1	1/15
3	LLASGIT	2.3	2	1/15

* *f*, Frequency (number of positive clones divided by total number of clones).
‡ Underlined sequences were observed by inverse and family PCR.

larged clonal CD8+ T cell populations in normal individuals should be considered in the interpretation of clonal T cell populations in disease states. Hence, evidence obtained by PCR alone for the presence of a clone of T cells at the site of an immune response is insufficient to link the T cells to the pathogenesis of the disease (18). To learn whether the clonally expanded TCR that we observed by PCR are pathogenetically relevant, we studied muscle sections from the same patient by immunohistochemistry.

In PM, two major populations of CD8+ T cells can be

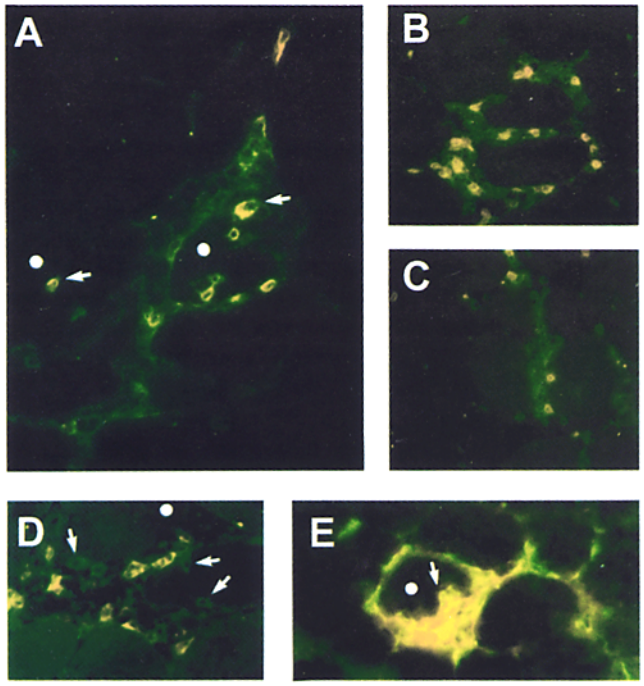

Figure 1. Localization of CD8 and TCR Vβ elements by double-fluorescence immunohistochemistry in three PM patients (*A–C*, patient 1; *D*, patient 2; *E*, patient 3). (*A*) Colocalization of CD8 (*green*, FITC) and Vβ13.1 (*red*, Cy3), patient 1. $CD8^+$ $V\beta13.1^-$ T cells are shown in green, $CD8^+$ $V\beta13.1^+$ T cells in yellow, and $CD8^-$ $V\beta13.1^+$ T cells in red. Two adjacent muscle fibers (*white dots*) are surrounded and invaded by T cells. Two examples of $CD8^+$ $V\beta13.1^+$ T cells are marked with white arrows. The fiber on the right is invaded by at least three additional $CD8^+$ $V\beta13.1^+$ T cells. The fiber on the left is invaded by at least one additional T cell that stained green, indicating that it is $CD8^+$ but $V\beta13.1^-$. Note red ($CD8^-$ $V\beta13.1^+$) cell at the top of the figure. ×250. (*B*) Colocalization of CD8 (*green*, FITC) and Vβ13.1 (*red*, Cy3), patient 1. Two adjacent muscle fibers are surrounded but not deeply invaded by T cells. The yellow cells are $CD8^+$ $V\beta13.1^+$. Note heterogeneity of the infiltrate. ×125. (*C*) Colocalization of CD8 (*green*, FITC) and Vβ5.1 (*red*, Cy3) in patient 1. A small number of labeled (*yellow or red*) $V\beta5.1^+$ cells are present among a majority of green ($CD8^+$ $V\beta5.1^-$) cells. ×125. (*D*) Colocalization of CD8 (*green*, FITC) and TCR Vβ5.1 (*red*, Cy3) in muscle of patient 2. Double-positive cells appear yellow. Two muscle fibers are invaded by $CD8^+$ $V\beta5.1^-$ (*green*) T cells (*arrows*). The upper fiber (*dot*) is only superficially invaded. ×250. (*E*) Colocalization of CD8 (*green*, FITC) and TCR Vβ3 (*red*, Cy3) in the muscle of patient 3. Note cluster of CD8, TCR-Vβ3 double-positive (*yellow*) T cells that invade a muscle fiber (*dot*). The majority of noninvasive (interstitial) T cells are $V\beta3^-$ (*green*). ×250.

Table 2. *Quantitative Immunohistochemical Analysis of Inflammatory T Cells (Patient 1)*

(A) T cells in muscle lesions

Lesion no.	CD8/Vβ13.1 +/−	CD8/Vβ13.1 +/+	CD8/Vβ13.1 −/+	Lesion no.	CD8/Vβ5.1 +/−	CD8/Vβ5.1 +/+	CD8/Vβ5.1 −/+
1	53	20	4	7	27	3	0
2	21	10	0	8	28	11	2
3	15	10	0	9	30	5	0
4	12	13	1	10	39	4	0
5	31	12	0	11	28	8	0
6	23	7	1	12	45	6	0
Σ	155	72	6	Σ	197	37	2

(B) Autoinvasive T cells in muscle fibers

Lesion no.	CD8/Vβ13.1 +/−	CD8/Vβ13.1 +/+	CD8/Vβ13.1 −/+	Lesion no.	CD8/Vβ5.1 +/−	CD8/Vβ5.1 +/+	CD8/Vβ5.1 −/+
1	0	0	0	7	0	1	0
2	2	2	0	8	1	0	0
3	1	2	0	9	2	0	0
4	1	2	0	10	6	0	0
5	3	6	0	11	5	1	0
6	2	0	0	12	5	0	0
Σ	9	12	0	Σ	19	2	0

Six focal inflammatory lesions (1–6) were analyzed by paired immunofluorescence for CD8 and Vβ13.1 (left columns), and six lesions (7–12) for CD8 and Vβ5.1 (right columns). Three different phenotypes were distinguished: $CD8^+V\beta^-$ (+/−), $CD8^+V\beta^+$ (+/+), and $CD8^-V\beta^+$ (−/+). (*A*) Numbers of all cells stained with one or both mAbs. 72/(155 + 72) = 32% of the $CD8^+$ cells were $V\beta13.1^+$ and 37/(197 + 37) = 16% were $V\beta5.1^+$. (*B*) Numbers of autoinvasive cells stained with one or both mAbs. 60% [12/(9 + 12)] of the autoinvasive $CD8^+$ T cells were $V\beta13.1^+$ and 10% [2/(19 + 2)] were $V\beta5.1^+$.

distinguished morphologically: (*a*) autoinvasive T cells that penetrate into muscle fibers; and (*b*) noninvasive T cells that are located in interstitial areas (1–3). We screened autoinvasive and noninvasive T cells for TCR expression. Using mAbs specific for Vβ13.1, Vβ5.1, or CD8 and double-fluorescence immunohistochemistry, we identified T cells with the predicted TCR Vβ proteins in situ (Fig. 1, *A–C*) and counted the cells expressing different phenotypes in 12 focal inflammatory lesions (Table 2). Within the total population of $CD8^+$ T cells, 32% were $V\beta13.1^+$ and 16% were $V\beta5.1^+$ (Table 2). In contrast, in the subpopulation of autoinvasive $CD8^+$ T cells, ~60% were $V\beta13.1^+$ and 10% were $V\beta5.1^+$ (Table 2). These immunohistochemical results confirm the presence of $V\beta13.1^+$ and $V\beta5.1^+$ T cells in the inflammatory lesions, and they indicate that the clonally expanded $V\beta13.1^+$ $CD8^+$ T cells preferentially invaded muscle fibers.

Using a series of different anti-TCR Vβ mAbs (see Materials and Methods) for immunohistochemical screening of muscle biopsy sections, we identified two additional patients who showed a striking accumulation of $CD8^+$ T cells staining for a particular Vβ element. In patient 2, 50% of the T cells were $V\beta5.1^+$. As in patient 1, the $V\beta5.1^+$ T cells were mainly located in interstitial areas (Fig. 1 *D*). However, in contrast to patient 1, the autoinvasive T cells could not be stained with anti-Vβ5.1, anti-Vβ13.1, or any other of the available anti-TCR mAbs (not shown). No RNA was avail-

able for TCR sequence analysis in patient 2, so that we could not determine the extent of clonal diversity of the interstitial $V\beta5.1^+$ or autoinvasive $V\beta5.1^-$ T cells.

In another patient, patient 3, 50% of all T cells and >75% of the autoinvasive T cells stained with an mAb against $V\beta3$ (Fig. 1 *E*). Using a $V\beta3$-specific primer, we amplified, cloned, and sequenced 15 $V\beta3^+$ cDNA clones from this patient's muscle. Nine clones (60%) had identical sequences (Table 1 *C*). Of the remaining six clones, two pairs of sequences were also identical (Table 1 *C*). Thus, the results in patients 2 and 3 confirm the findings in patient 1, demonstrating an accumulation of $V\beta^+$ $CD8^+$ T cells, differential TCR usage by autoinvasive and noninvasive (interstitial) T cells, and clonal expansion of autoinvasive T cells.

Previous studies of the TCR repertoire in PM did not combine sequence with histological analysis of TCR $V\beta$ expression (21–23). Using family PCR analysis of needle biopsy specimens, one study (21) found preferential usage of $V\alpha1$, $V\alpha5$, $V\beta1$, and $V\beta15$, but no evidence for clonal expansion. By contrast, another report (22) described increased frequency of TCR $V\alpha1$ and $V\beta6$ and a high degree of clonality in the $V\beta6$ TCR family. The findings of preferential TCR V region usage (21, 22) and clonality (22) are consistent with our results. The differences in TCR usage observed in the different studies could relate to differences between patients (e.g., different HLA types).

The approach we have taken to study the TCR repertoire in PM differs in two major aspects from the earlier studies (21, 22, 23). First, we combined two independent PCR techniques, inverse and family PCR. Inverse PCR is not biased by the selection of V region–specific primers (9), and the combination of two PCR techniques helps to minimize artifacts. Second, we combined PCR analysis with the immunohistochemical localization of T cells. This approach allowed us to study the heterogeneity and topology of TCR usage in individual lesions.

We are aware that our data do not formally prove that the autoinvasive $CD8^+$ T cells actually destroy the muscle fibers. However, together with previous morphological (1–3, 24) and functional (25) observations, the results are consistent with the following mechanism of muscle fiber injury: some muscle fibers, which express MHC class I and MHC class I–associated auto-Ag(s) (1–3), are surrounded by $CD8^+$ T cells. Some of the T cells traverse the basal lamina of the muscle fiber, contact the muscle fiber surface, and migrate towards the center of the muscle fiber (1–3, 24). As shown by immunoelectronmicroscopy, the autoinvasive T cells extend spike-like processes into the surrounding cytoplasm of the muscle fiber (24). Eventually, the autoinvasive $CD8^+$ T cells may literally "honeycomb" the muscle fiber (24). Whether the muscle fiber degenerates by apoptosis or necrosis has not been established (1–3, 24).

In conclusion, our analysis demonstrates that the TCR $V\beta$ repertoire expressed in the muscle of PM patients is highly restricted and that different $V\beta$ elements may predominate in different patients. Furthermore, we found that there is a dissociation between the TCR usage of autoinvasive and noninvasive $CD8^+$ T cells, and that the autoinvasive T cells are clonally expanded. We hypothesize that the autoinvasive $CD8^+$ T cells are specific for the yet unknown auto-Ag(s).

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