Severe Perturbations of the Blood T Cell Repertoire in Polymyositis, But Not Dermatomyositis Patients

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Polymyositis and dermatomyositis are diseases characterized by muscle weakness and muscle inflammatory infiltrates. Their pathogenesis remains unclear. A central role for endomysial autoaggressive CD8⁺ T cells is suspected in polymyositis and for perivascular B cells in dermatomyositis. We compared the T cell repertoire of 10 polymyositis and 10 dermatomyositis patients by immunoscope, a method providing a global assessment of the T cell repertoire and a sensitive detection of clonal T cell expansions. Samples were analyzed qualitatively and quantitatively in the blood (unsorted cells and CD4⁺ and CD8⁺ cells) and in muscle infiltrates. Dramatic perturbations of the T cell repertoire were observed in the blood of polymyositis but not dermatomyositis patients (p < 0.0005), the latter being undistinguishable from controls. These perturbations were due to oligoclonal expansions of CD8⁺ T cells and most blood clonal expansions were also found in muscle. These results indicate that the pathogenesis of polymyositis and dermatomyositis is different and reinforce the view that polymyositis but not dermatomyositis is an autoimmune CDR3 T cell-mediated disease. Moreover, this method may be helpful for the differential diagnosis of polymyositis and dermatomyositis and for noninvasive follow-up of polymyositis patients. The Journal of Immunology, 2001, 167: 3521–3529.

Severe Perturbations of the Blood T Cell Repertoire in Polymyositis, But Not Dermatomyositis Patients

Olivier Benveniste,*† Patrick Chérin,*† Thierry Maisonobe,‡ Rastine Merat,§ Olivier Chosidow,† Luc Mouthon,¶ Loïc Guillevin,¶ Antoine Flahault,‖ Marie-Christine Burland,* David Klatzmann,* Serge Herson,†* and Olivier Boyer2*
By using the immunoscope approach, we observed severe perturbations of the peripheral T cell repertoire in the blood of PM but not DM patients. These perturbations occurred in the CD8+ sub-population and reflected a subset of the clonal expansions present in muscle infiltrates of PM patients. These results indicate that the pathogenesis of PM and DM is indeed different and support the view that PM but not DM is an autoimmune CD8+ T cell-mediated disease. They also open perspectives for a noninvasive TCR-based follow-up of PM patients.

Materials and Methods

Patients

Twenty consecutive patients with a diagnosis of PM (n = 10) or DM (n = 10) (Table I), and a group of healthy control subjects (n = 17, male: female = 7:10, age = 32 ± 9 years) were included in the study between December 1998 and February 2001. The institutional ethics committee approved the study and patients gave written informed consent. None of the patients had inclusion body myositis, cancer, or other connective tissue disorders. The criteria for diagnosis of PM and DM were based on studies by Bohan and colleagues (26, 27): symmetric muscle weakness, increased serum muscle enzyme, myopathic changes on electromyography, and typical histological findings on muscle biopsy and/or characteristic dermatological manifestations (heliotrope rash, periangual erythema, Gottron papules, and poikilodema) for DM. The diagnosis of PM or DM was considered definite in all cases since each patient presented at least four manifestations. All PM patients and 8 of 10 DM patients had a muscle biopsy. Since the diagnosis was based on the above-mentioned criteria, it was evident for DM04 and DM06, it was considered nonetohinal to perform muscle biopsy. Six of 10 PM and 7 of 10 DM patients were included at onset of their disease before any treatment. Four of 10 PM and 3 of 10 DM patients were included at relapse. The latter had previously been treated with prednisone (seven of seven) with or without immunomodulator agents (methotrexate, three of seven; i.v. Ig, two of seven; azathioprine, one of seven; or cyclosporine, one of seven). At inclusion, they either had no treatment (four of seven) or received only low-dose corticosteroids (prednisone ≤ 6 mg/day for at least 6 mo). Muscle weakness was evaluated using a modified grading scale of the British Medical Research Council: values from 0 to 11 were assigned to the strength of 8 muscles (neck flexors, trapezius, deltoid, biceps, psoas, maximus and medius glutaeus, quadriceps), yielding a theoretical maximum score of 88 points (28). Age, disease duration, muscle strength, and creatine kinase levels were not statistically different between PM and DM patients.

Biological samples

PBMC were separated on a Ficoll-Hypaque gradient (density 1.077 g/ml), washed in RPMI medium, and resuspended in sterile PBS. A minimum of 7 × 10^6 PBMC was used for RNA extraction. Available samples of liquid nitrogen-frozen muscle biopsies performed for diagnostic purposes in PM patients were used for RNA extraction and further repertoire analysis using BV-BC PCR amplification and BJ-primed run-off reactions.

TCR CDR3 size analysis

Experiments were performed as described elsewhere (19, 29, 30). Analysis was performed in 14 different BV families that cover >70% of the T cell repertoire of healthy subjects (31). Briefly, cellular RNA was reverse transcribed into cDNA using oligo(dT) and Moloney murine leukemia virus reverse transcriptase (RT) (Life Technologies, Rockville, MD). After phenol/chloroform extraction, a quantity of cDNA corresponding to 300 ng of total RNA was amplified by PCR in a 50-μl reaction using one primer for each of the 14 BV studied and a common BC primer (Genset, Paris, France). The final concentration was 0.5 μM for each primer, 0.2 mM dNTP (except for 0.4 mM dUTP), 2 mM MgCl 2 in PCR buffer (Boehringer Mannheim, Mannheim, Germany) in the presence of 2.5 U of Taq polymerase (Boehringer Mannheim). The amplification was performed on a thermal cycler (Thermo Hybird, Ashford, U.K.) with an initial denatur- ation step at 94°C for 5 min, then 40 of the following cycles: 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final step at 72°C for 10 min. Amplification was verified by agarose gel electrophoresis. Each BV-BC PCR product was subjected to 10 run-off cycles primed with a nested fluorophore-labeled BC or a BJ primer in the presence of 1 U of Taq polynucleotide. Each run-off product was denatured and loaded on a gel for fluorescence analysis using an Applied Biosystems 377 sequencer (PerkinElmer, Norwalk, CT). Raw data were analyzed with Immunoscope 3.01b software (Loginsperm, Paris, France) (19).

The method for quantifying T cell repertoire perturbations was adapted from Ref. 32. Briefly, CDR3 length profiles are translated into a p distribution p(i) for each peak i (i = 1–8) of a given BV family as a function of the area under the curve (expressed in relative fluorescence intensity, RFI) with normalization so that Σp(i) = 100%. The extent of perturbation D(i) for each peak i is computed by the distance D(i) between p(i) values from the sample and pRFi values from a reference distribution derived from repertoire analysis of eight cord blood samples. The perturbation D(i) for a given BV family is then calculated as ΣD(i)/2, so that D = 0 or 100% for a p distribution equal to or completely nonoverlapping with the reference, respectively. The mean of individual BV family perturbations yields the average perturbation D = ΣD/n for all n BV families (n = 14). Quality assurance of data processing was provided by parallel data entry by two independent investigators followed by separate calculations using two independently programmed Excel spreadsheets (Microsoft, Redman, WA).

FACS purification of T cell subsets

PBMC were incubated with FITC-labeled anti-CD8 and PE-labeled anti-CD4 mAbs (Coulter Immunotech, Marseille, France), washed, filtered through a nylon Falcon 2350 cell strainer (BD Biosciences, Mountain View, CA) and sorted using a FACStar® Plus (BD Biosciences). Sorted CD4+ and CD8+ cells were recovered in RPMI medium supplemented with 50% FCS. Cells were then washed in PBS and placed in RNAble for RNA extraction and further T cell repertoire analysis. The purity of sorted cell populations was always >98%.

Statistical analyses

Statistical analyses were performed using Statview software (SAS Institute, Cary, NC). The Mann-Whitney U test was used to compare data. The confidence interval for specificity and sensitivity was computed with correction for continuity (33, 34).

Results

Qualitative analysis of the blood T cell repertoire in PM and DM patients

Using the immunoscope method, the distribution of TCR β CDR3 lengths is visualized as a series of peaks separated by a distance of three nucleotides corresponding to in-frame transcripts. A physiologically diverse repertoire yields a gaussian-like profile, whereas the presence of T cell clonal expansions manifests as a distribution skewed by the accumulation of larger peaks. We studied the blood T cell repertoire of 10 PM and 10 DM patients. Most of them (17 of 20) were not receiving any treatment at the time of inclusion while a minority (3 of 20) were receiving only very low-dose prednisone (Table I).

All PM patients exhibited severely perturbed CDR3 distributions (Fig. 1). One or more discrete peaks accumulated within different BV families of each PM patient. These T cell expansions appeared to be oligoclonal and, for a given patient, occurred in several BV families. From one patient to another, these expansions did not concern the same BV and no recurrence of a particular peak was found. In marked contrast, all patients with DM presented a gaussian-like distribution in the vast majority of their BV families (Fig. 2). Nevertheless, in a minority of DM patients, some expansions were occasionally observed in a few BV families.

Quantitative analysis of the T cell repertoire

To perform statistical analyses, we quantified the extent of perturbation of the T cell repertoire. For this, we first established reference values for the CDR3 length p distribution of each BV family in eight cord blood samples. Cord blood was chosen because it provides a representation of the theoretical unperturbed repertoire of naive preimmune T cells (35). The extent of perturbation, D, for each of the BV families of a given sample, is calculated as a function of the difference between the p distribution of this sample and the reference p distribution (Fig. 3a). Furthermore, the average perturbation D can be calculated as the mean of the D values of the 14 BV families studied. This approach provides an objective
and quantitative determination of repertoire perturbations with \( D \) values varying from 0 (absence of perturbation) to 100% (theoretical maximum perturbation).

\( D \) values of PM patients were significantly higher than those of DM patients (\( p < 0.0005 \)). In contrast, \( D \) values of DM patients were undistinguishable from those of a series of 17 healthy controls (Fig. 3b). Furthermore, there was no overlap in the distribution of \( D \) values from PM and DM patients (range, 11.4–20.9% for PM and 4.4–10.7% for DM). Thus, a threshold \( D_{\text{thres}} = 11\% \) could be defined which allowed to distinguish PM and DM with an estimated sensitivity and specificity of 100% (confidence interval, 66–100%).

At the individual BV level, statistically significant perturbations of the repertoire of PM patients as compared with DM patients were observed in 12 of 14 BV families (Fig. 3c). In BV2 and BV15 families, although the difference did not reach statistical significance, there was a trend toward a higher level of perturbation in PM as compared with DM patients. Together, the results of qualitative and quantitative repertoire analyses strongly suggest that the physiopathological process underlying PM and DM is different, with a central role for clonally expanded T cells in PM but not in DM.

**Repertoire analysis of blood CD4\(^+\) and CD8\(^+\) T cell subsets**

The simplest explanation for the results observed in PM patients is that cytotoxic T cells responsible for muscle cell injury recirculate from muscle to blood where they can be detected by the immunoscope method. This implies that the observed repertoire perturbations should be primarily due to clonal expansion of CD8\(^+\) T cells, since this subset is largely predominant in muscle lesions (8, 16, 17). To test this hypothesis, we studied the repertoire of sorted CD4\(^+\) and CD8\(^+\) T cells in three patients for whom sufficient numbers of PBMC were available (PM-05, PM-06, and PM-09). This analysis revealed that the perturbations observed in unsorted PBMC were due to clonal expansions of CD8\(^+\) but not CD4\(^+\) T cells (Fig. 4a). In the CD8\(^+\) subset, the average perturbation \( D \) reached the high value of 37.3 ± 1.3%, which was significantly greater than that in unsorted T cells (Fig. 4b). In contrast, in the CD4\(^+\) subset, \( D \) values were significantly lower than in unsorted T cells and, notably, remained inferior to \( D_{\text{thres}} = 11\% \). Thus, blood T cell repertoire perturbations in PM patients are due to the clonal expansion of CD8\(^+\) T cells.

**T cell repertoire analysis in muscle cellular infiltrates of PM patients**

The presence of clonally expanded CD8\(^+\) T cells in the blood of PM patients suggests that they belong to the same population of autoaggressive T cells that cause muscle injury. We therefore aimed to determine whether the blood T cell expansions were due to recirculation of muscle-infiltrating T cells. For this, we analyzed the repertoire of muscle infiltrates in four PM patients for whom a frozen biopsy was available and compared it to their blood repertoire. Since two different clones using the same BV gene segment can fortuitously share the same CDR3 length, which would therefore yield undistinguishable peaks, analyses were performed at the BV-BJ rather than the BV-BC resolution so as to better ascertain clonal identity.

This comparative analysis revealed that most of the clonal expansions observed in the blood of PM patients corresponded to peaks also found in their muscle infiltrates. For example, this was the case for the BV3-BJ2.2 peak in PM-01, the BV1-BJ2.7 peak in PM-04, the BV15-BJ2.5 peak in PM-06, and the BV15-BJ2.1, BV15-BJ2.3, and BV15-BJ2.7 peaks in PM-08 (Fig. 5). Nevertheless, some peaks present in muscle infiltrates could not be detected in the blood. For instance, this was the case for the left BV3-BJ1.5 peak in PM-01 muscle or the right BV1-BJ2.5 peak in PM-04 muscle which were not found in the blood (Fig. 5). Finally, in some instances a peak present in blood was not found in muscle, such as the right BV15-BJ2.3 peak in PM-06, but this situation was rare and it is presumable that such peaks correspond to clonal expansions unrelated to the PM disease. For PM-04 and PM-06, the blood sample was obtained at the time of diagnosis. It should be noted that the blood T cell repertoire was determined at relapse in PM-08 and was compared with that of a previous muscle biopsy.

### Table 1. Patient characteristics

<table>
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<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Duration (mo)</th>
<th>Relapse Duration (mo)</th>
<th>Previous Treatment</th>
<th>Current Treatment</th>
<th>Muscle Testing</th>
<th>Skin Lesions</th>
<th>CK (U/L)</th>
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<td>61/F</td>
<td>12</td>
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<td>61</td>
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<td>47/M</td>
<td>38</td>
<td>4</td>
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<td>Pred, 6 mg/day</td>
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<td>Pred, MTX</td>
<td>60</td>
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<td>68</td>
<td>PK, GP</td>
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<td>53</td>
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<td>PK, HR</td>
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<td>HR, PE, GP, PK</td>
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<td>HR</td>
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*CK, Creatine kinase (normal <160 U/L); Pred, prednisone; MTX, methotrexate; Aza, azathioprine; CsA, cyclosporine A; HR, heliotrope rash; PE, perungual erythema; GP, Gotron papules; PK, poikiloderma; F, female; M, male; ND, not done.

*Muscle testing: values from 0 to 1 were assigned to the strength of 8 muscles, yielding a theoretical maximum score of 88 points.
performed at the time of diagnosis, i.e., 33 mo earlier. Strikingly, the peaks detected in the blood at relapse were identical to those detected earlier in muscle. Similarly, several clonal expansions persisted over a 7-mo period in PM-01 (Fig. 5). Together, these results indicate that the repertoire perturbations observed in the blood of PM patients reflect part of the clonal expansions present in the muscle infiltrates and suggest a long-term persistence of repertoire anomalies in PM.
Discussion

Identifying target autoantigens and developing methods to quantify and trace autoaggressive T cells in affected individuals are major goals in autoimmunity (36). In particular, whereas several autoimmune diseases are associated with the presence of autoantibodies, clinically useful blood markers of autoimmune disease-associated T cells are lacking. Here, we demonstrate that PM is associated with dramatic perturbations of the T cell repertoire that can be readily observed in the patients’ peripheral blood. These perturbations are due to the oligoclonal expansion of CD8+ T cells and reflect the recirculation of muscle-infiltrating T cells. To our knowledge, this is the first report of a noninvasive method to monitor autoimmune disease-associated T cells directly in the blood of human patients.

FIGURE 2. Immunoscope analysis of the T cell repertoire in DM patients (see Fig. 1 legend).
FIGURE 3. Quantitative analysis of T cell repertoire perturbations in PM and DM patients. a, Method for quantifying the T cell repertoire, as adapted from Ref. 32: example of BV4 from PM-03. The CDR3 length profile is translated into a p distribution $p_{BV4}(i)$ for each peak $i$ ($i = 1–8$) as a function of the area under the curve, with normalization so that $\sum p_{BV4}(i) = 100\%$. The extent of perturbation $D_{BV4}(i)$ for each peak $i$ is computed by the distance between $p_{BV4}(i)$ values from the sample and $p_{ref_{BV4}}(i)$ values from a reference distribution obtained from analysis of eight cord blood samples (mean $\pm$ SEM). The perturbation $D_{BV4}$ is then calculated as $\sum |D_{BV4}(i)|/2$. b, Global comparison of T cell repertoire perturbations. The mean of $D_{k}$ perturbations for all $n_{BV_k}$ families ($n = 14$) yields the average perturbation $D = \sum_{k} D_{k}/n$. Average perturbations $D$ are computed for a group of healthy volunteers (control (Ctrl), $n = 17$), and the DM (○) and PM (●) patients. Numbers are mean $\pm$ SEM for each group. The difference in average perturbations $D$ between DM and PM patients is statistically significant (Mann-Whitney $U$ test), whereas the difference between the control and the DM groups is not. This analysis revealed a threshold $D_{\text{thres}} = 11\%$ discriminating the $D$ value distribution of PM patients from that of DM patients (dotted line). c, Comparison of T cell repertoire perturbations for each BV. Individual perturbations $D_{k}$ for each $BV_k$ are compared between DM (○) and PM (●) patients (Mann-Whitney $U$ test).

FIGURE 4. Immunoscope analysis of the T cell repertoire in CD4$^+$ and CD8$^+$ T cell subsets of three PM patients. CD4$^+$ and CD8$^+$ T cells were purified by FACS. Purity of sorted cell populations was >98%. a, Comparison of immunoscope profiles in three representative BV families where T cell expansions were observed in the unsorted T cell population. Horizontal and vertical axes represent the size of the amplified product (nucleotides) and RFI, respectively. b, Quantification of T cell repertoire perturbations. Numbers are mean $\pm$ SEM. Average perturbations $D$ in CD4$^+$ and CD8$^+$ subsets were significantly different from those of unsorted T cells (Mann-Whitney $U$ test). The difference between average perturbations of CD4$^+$ and CD8$^+$ subsets was also statistically significant.
In contrast to normal muscle tissue, myocytes of PM patients express HLA class I molecules at their surface (37–39), allowing the presentation of muscular autoantigens to cytotoxic CD8+ T cells. Furthermore, it was recently reported that forced expression of MHC class I molecules in skeletal muscle of young mice induces a PM-like disease accompanied by mononuclear cell muscle infiltrates (40). In the present study, multiple CD8+ T cell expansions were found in PM patients, suggesting that these cells may target several autoantigens, even at an early stage of the disease. Previous studies had already observed the presence of in situ clonally expanded T cells in muscle of PM patients. Notably, a histochemical analysis using mAbs revealed the overrepresentation of Vα2 and Vβ3 TCRs within the endomysium (13). Also, studies based on PCR analyses other than immunoscope have reported different T cell repertoire biases: overusage of Vα1, Vα5, Vβ1, and Vβ15 (12), Vα1 and Vβ6 (14), or Vα33, Vβ5, and Vβ13 TCRs (15). The T cell expansions identified in the present study did not preferentially occur in selected BV families but rather differed from one patient to another, in accordance with these previous reports. MHC polymorphism may partly account for the diversity of this T cell response since patients were not selected for HLA groups.

We found that most blood T cell expansions were also detected in the muscle infiltrates. In contrast, some muscle-infiltrating T cells were not detected in the blood, suggesting that only a subset of these cells actually recirculates outside the target tissue. The physiopathological significance of this finding remains to be established. It might be hypothesized that the expansions found in the blood mainly reflect the T cells causing muscle injury. Nevertheless, it cannot be ruled out that some truly aggressive T cell clones do not recirculate because they find high-affinity ligands in muscle. We are aware that our data do not formally prove that the expanded T cells clones found in muscle and blood of PM patients are actually muscular autoantigen-specific cytotoxic T cells. Conclusions on the functional importance of expanded clones to the disease process will benefit from further isolation of these T cells and testing them in functional assays. Nevertheless, along with previous morphological observations (1, 41, 42) and the demonstration that T cell lines from PM patients can be cytotoxic against autologous myotubes (43), our results strongly argue for a muscle-specific T cell-dependent autoimmune mechanism in PM. In support of this, we found that the blood T cell clones which had expanded in vivo responded to IL-2 in cell culture (data not shown), suggesting that they might be effector cells that had been primed in vivo, presumably upon recognition of muscular autoantigens.

When we analyzed the blood BV usage by FACS, a technique that provides quantitative data on the T cell repertoire through only 20 parameters, we were unable to discriminate PM patients from DM patients or healthy controls (data not shown). This indicates that FACS analysis underestimates the extent of repertoire perturbations as compared with immunoscope, in agreement with a recent report performed in HIV-infected patients (44). Using sequencing of PCR-amplified products, Bender et al. (15) demonstrated the presence of multiple T cell clonal expansions in muscle infiltrates. Among these expansions, three TCR sequences were searched for in the blood but could not be found by the authors. Thus, when compared with other methods, the immunoscope approach, which analyzes TCR diversity through 100-2000 parameters (24), allows a global estimation of the T cell repertoire.
and a highly sensitive detection of overrepresented T cell populations.

In marked contrast to PM patients, the level of repertoire perturbations in DM patients remained undistinguishable from that of healthy controls. Rarely, some skewed distributions were observed in a minority of BV families, but this most likely reflects the occurrence of clonal expansions that physiologically accumulate with age (45–48). Thus, the present results strongly argue for essential differences in the pathogenesis of these two inflammatory myopathies, with a central role for CD8+ T cell-mediated autoimmunity in PM but not DM. It can be argued that anti-synthetase autoantibodies may be present in PM patients while some CD8+ T cells can be found in the inflammatory infiltrates of DM patients. Nevertheless, the frequency of PM patients with anti-synthetase autoantibodies is low (49, 50) and the presence of T cell muscle infiltrates in DM patients does not lead to detectable repertoire anomalies in the blood. Furthermore, since quantitative repertoire analysis allowed us to discriminate PM and DM with high sensitivity and specificity, this approach may potentially be helpful in some cases for the differential diagnosis between PM and DM. So far, the immunoscope method is still relatively labor intensive, but it is likely that the development of more automated systems such as capillary sequencers will simplify repertoire analysis and allow a more routine use in the near future.

Relapse is frequent in the course of PM, notably during the period of corticosteroid dose de-escalation. Prediction of PM recurrence remains challenging because follow-up is based mainly on clinical examination (repeated muscle weakness scales) and creatine kinase levels, all of which worsen concomitantly with relapse. Furthermore, these parameters reflect the consequences rather than the cause of muscle cell injury. The ability to trace autoaggressive T cells in the blood opens perspectives for monitoring repertoire anomalies under immunomodulatory treatments and the possibility of correlating their evolution with clinical status. If it is found that a disappearance or decrease of T cell perturbations correlates with recovery and a reappearance or increase of relapse, immunoscope analysis would provide a helpful tool for follow-up and prediction of recurrence in PM patients. In this respect, the cases of patients PM-01 and PM-08 already contribute some preliminary evidence that the T cell perturbation at relapse may be similar to that found earlier at diagnosis. Finally, the present approach may also facilitate further identification of the still unknown T cell autoantigen(s) in PM.

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References


