Clonal expansion of CD8+ BV8 T lymphocytes in bone marrow characterizes thymoma associated B-lymphopenia.

By

Anna Maria Masci # §, Giovannella Palmieri *, Laura Vitiello #, Liliana Montella *, Francesco Perna **, Paola Orlandi^, Gianfranco Abbate §, Serafino Zappacosta #, Raffaele De Palma §, and Luigi Racioppi #.

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# Dipartimento di Biologia e Patologia Cellulare e Molecolare, Federico II University of Naples, Italy; * Dipartimento di Oncologia molecolare e clinica, Federico II University of Naples, Italy

** Laboratorio di Microbiologia e Chimica Clinica, Monaldi Hospital, Naples, Italy;

^ Divisione di Immunologia e Malattie Infettive, Ospedale Bambin Gesù, “Tor Vergata” University of Rome, Rome, Italy; § Dipartimento di Inernistica Clinica e Sperimentale, Second University of Naples, Naples, Italy.

Correspondence to: Dr Luigi Racioppi, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Federico II University of Naples, Italy; 5 via S. Pansini, I-80131 Naples, Italy. Phone:

+39-81-7463311; Fax +39-81-7463252. E-mail: racioppi@unina.it.

M.A.M. an P.G. made an equal contribution to this study.
Summary

A subgroup of thymoma patients is affected by severe immunodeficiency clinically resembling an HIV infection (Good’s Syndrome). These individuals are characterized by B-lymphopenia with B-lymphopoiesis deficiency. To investigate the pathogenesis of this unique condition, we studied T-cell repertoire in blood and bone marrow samples by heterogeneity length analysis of CDR3 beta variable regions of the T-cell receptor (Spectratyping). While no alterations were found in the peripheral blood, we detected an oligoclonal population of BV8 CD8+ T-cells in five out of five bone marrow samples. No lymphocyte expansions were found in the bone marrow of two thymoma patients with normal B-cell counts, two healthy donors and three patients with thymoma-unrelated diseases. These data suggest that an immune response toward an unknown antigen is taking place in the marrow of B-lymphopenic thymoma patients. We propose that BV8 CD8+ T cells may play a role in the pathogenesis of this immunodeficiency syndrome.
Introduction

Thymomas are tumors of thymic epithelium characterized by severe alterations of the immune system not yet completely understood (1-8). A severe immunodeficiency with B-cell defect is found in a small percentage of thymoma patients (1-6), this condition is also known as Good’s Syndrome (2). The immunodeficiency may precede or follow thymectomy and B-cells are undetectable in the periphery (4, 9, 10). The coexistence of autoimmunity and immunodeficiency causes life-threatening problems in the management of these patients (7-10).

The pathogenesis of B-cell loss represents a puzzling phenomenon, the understanding of which might help us to find a better approach to treat this syndrome. It has been documented that CD8+ T-lymphocytes derived from blood of patients with thymoma and immunodeficiency are able to suppress proliferation of allogeneic pre-B cells. Thus, it has been suggested that an aggression toward B cell precursors is responsible for the immunodeficiency (4-6). The Spectratyping analysis represents a novel tool to address this unresolved question (11-15). Therefore, we examined the T cell repertoire of peripheral and bone marrow lymphocytes in five thymoma patients suffering from immunodeficiency and in seven control individuals. The absence of B lymphocytes was always characterized by the expansion of BV 8 positive T cells in the marrow. These data provide novel evidences for the involvement of CD8+ T cells in the pathogenesis of Good’s Syndrome, supporting the idea that B cell loss is due to an aggression toward B cell precursors.

Study Design

The main characteristics of the patients included in this study are shown in table 1. Tumor hystology has been classified according to WHO proposal (16). The majority of them received thymectomy and showed an inversion of the CD4+/CD8+ ratio. Five patients were suffering from severe immunodeficiency syndrome characterized by recurrent infections of the lower respiratory tract. Flow cytometry analyses were performed at monthly intervals in the last six months showing a stable reduction of mature CD19+ peripheral cells (Unique Patient Number, UPNs 091, 157, 051, 009, 147). A marked hypogammaglobulinemia had been detected in four out of five patients, while one of them has normal Ig serum levels (UPN 091). On the bases of clinical signs and B lymphopenia we classified these patients as suffering from Good’s syndrome. We included in the study also two thymoma patients with normal Ig serum levels, normal B cell counts and no signs of severe recurrent infections (TC 011, TC 087). Three patients with thymoma unrelated diseases (SLE, Myelodysplasia, and Histiocytosis, SLE 354, MD 27 and Hys 104 respectively) as well as two healthy donors (HC 1 and HC 2) were also
investigated. In the year before BM sampling patients 009 and 147 received Prednison and Cyclosporin. A. Treatments were suspended one month before bone marrow sampling. Thus, we considered all patients free of immunosuppressive treatment at the moment of this study. All subjects were fully informed about the aim of the research and agreed to donate samples. Marrow samples were obtained taking advantage of material used for diagnostic purposes. Morrow samples from two allogenic transplantation donors were used as controls (healthy controls: HC 1 and HC 2). The immunophenotype was done on whole blood by means of standard three-color flow cytometry. To perform the Spectratyping (11), T-cells were separated from blood and marrow by density gradient according to standard procedures (Lymphoprep, Oslo, Norway). When a sufficient amount of lymphocytes was available from marrow samples, T cells were separated in CD8+ and CD8- cells by magnetic sorting with coated beads (Dynabeads, Dynal, Oslo, Norway). RNA was prepared by Trizol™ (GIBCO-BRL, Bethesda, MD) according to the manufacturer’s instructions. Reverse-transcription and PCR amplification for the 25 different beta variable TCR families (BV) was performed by the one-step PCR protocol (GIBCO-BRL, Bethesda, MD). To resolve the composition of each BV TCR family, the PCR products were run on a sequencing gel in fluorescence based DNA sequencer (11-13). Normally, each TCR family is resolved by this technique as a series of bands having a gaussian distribution. Each alteration, in either the distribution or the intensity of single bands, represents a perturbation in the given BV-TCR family reflecting the involvement of that TCR BV family in an immune response toward one or more antigens (11-15). PCR products corresponding to BV8 regions showing a single peak were separated on polyacrylamide gel. The major band was than cut from the gel, eluted and sequenced by direct PCR method (Big Dye Sequencing kit, V3.0, Applied Biosystem, Warrington, UK).

Results and Discussion

The immunophenotype analysis of peripheral lymphocytes derived from thymoma patients with immunodeficiency showed a stable decrease in the level of mature CD19+ (Tab. 1) and CD20+ B cells (data not shown). Unexpectedly, the BV 8 family showed a single-peak profile in bone marrow, but not in peripheral blood lymphocytes in five out of five B-lymphopenic patients (Fig 1A). We reasoned that these BV 8 families could share similarity in the CDR3 sequences, too. The direct sequencing analysis confirmed this hypothesis, revealing a conserved CDR3 motif (SF/LGXGXNXXQ/LH/Y) in all BV8 products found to be expanded (Fig 1B). T cells isolated from marrow and blood samples of TC 087 patients and from the other control individuals expressed detectable amount of BV8 mRNA with a gaussian CDR3 profile (Fig. 1 C). In the patient TC 011 we found only a barely detectable level of BV8 mRNA in marrow-derived lymphocytes (Fig. 1A). In the figure 1D, we report a synopsis of the whole repertoire analysis expressed in CD8+ and CD8- T cells. A very limited number of altered
families can be found in the marrows of B lymphopenic patients (Fig 1D). Noteworthy, only BV8 families display a single-peak profile. In two out of three patients BV8 have been found expanded only in CD8+ subset (UPN 091 and 157). In the individual 147, BV8 was expanded in CD8+ as well as in CD8- T cells. However, in this case the expression level was much higher in CD8+ than in CD8- lymphocytes (data not shown).

In conclusion, the whole body of data confirmed that bone marrow of B-lymphopenic thymoma patients was infiltrated by the oligoclonal expansion of CD8+ BV8+ T-lymphocytes. Since in the periphery BV8+ lymphocytes were normal, it was likely that the selective expansion detected in the marrow would results from an immune response toward an unknown pathogen (i.e. B lymphotropic virus) or from a direct autoimmune aggression against B-cell precursors. Autoimmune reaction against haematopoietic precursors seems to be responsible for disorders that are associated or not with thymoma (17-20). Clonal lymphocyte expansion has been found in the peripheral blood of patients with Pure Red Cells Aplasia (17-19). Furthermore, the analysis of the CDR3 size distribution in the bone marrow of patients with Aplastic Anemia indicates that an antigen-driven expansion of T cells is involved in the pathogenesis of this disease (20). Our findings provide novel evidence supporting the hypothesis that a T cell-mediated reaction against haematopoietic precursors is responsible for the selective B-cell loss in a subgroup of thymoma patients. In light of this, the immunodeficiency with severe B lymphopenia syndrome associated with thymoma has to be included in the autoimmune phenomena occurring in the patients affected by this form of tumor. Further experiments are warranted to characterize the (auto)antigen(s) possibly involved in triggering this phenomenon.

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References


Table 1. Clinical and immunological findings in patients with thymoma

<table>
<thead>
<tr>
<th>Patients (UPN)</th>
<th>Sex/Age</th>
<th>Histology/Stage</th>
<th>Autoimmune Associated Disease</th>
<th>Thymectomy (BM sampling)</th>
<th>CD3+</th>
<th>CD3+ CD4+</th>
<th>CD3+ CD8+</th>
<th>CD19+</th>
<th>I gG</th>
<th>I gA</th>
<th>I gM</th>
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<tr>
<td>091</td>
<td>M/56</td>
<td>B2/IVa</td>
<td>no</td>
<td>1995 (1999)</td>
<td>1915</td>
<td>319</td>
<td>1482</td>
<td>0</td>
<td>7.4</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>157</td>
<td>M/45</td>
<td>nd/IV</td>
<td>no</td>
<td>1982 (1999)</td>
<td>786</td>
<td>266</td>
<td>484</td>
<td>0</td>
<td>1.9</td>
<td>0.2</td>
<td>0.2</td>
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<tr>
<td>051</td>
<td>F/70</td>
<td>C/III</td>
<td>No</td>
<td>No (1999)</td>
<td>697</td>
<td>252</td>
<td>436</td>
<td>25</td>
<td>1.2</td>
<td>0.3</td>
<td>0.1</td>
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<td>009</td>
<td>F/52</td>
<td>AB/II</td>
<td>MG/A</td>
<td>1995 (1999)</td>
<td>2115</td>
<td>910</td>
<td>951</td>
<td>68</td>
<td>1.8</td>
<td>0.3</td>
<td>0.1</td>
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<td>147</td>
<td>M/28</td>
<td>nd/IVa</td>
<td>PRCA</td>
<td>1998 (1999)</td>
<td>1185</td>
<td>402</td>
<td>462</td>
<td>58</td>
<td>4.2</td>
<td>0.8</td>
<td>0.3</td>
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<td><strong>Thymoma patients without B lymphopenia</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.C. 011</td>
<td>F/58</td>
<td>B1/II</td>
<td>no</td>
<td>1995 (1999)</td>
<td>750</td>
<td>165</td>
<td>240</td>
<td>292</td>
<td>8.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>T.C. 087</td>
<td>M/48</td>
<td>B3/IVa</td>
<td>no</td>
<td>1998 (1999)</td>
<td>841</td>
<td>168</td>
<td>543</td>
<td>170</td>
<td>10</td>
<td>2.4</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Control patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hys 104</td>
<td>F/44</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a. (2002)</td>
<td>1970</td>
<td>1131</td>
<td>374</td>
<td>796</td>
<td>12</td>
<td>3.4</td>
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<tr>
<td>SLE354</td>
<td>F/38</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a. (2002)</td>
<td>2890</td>
<td>1970</td>
<td>750</td>
<td>1320</td>
<td>32</td>
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</tr>
<tr>
<td>MD27</td>
<td>M/24</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a. (2002)</td>
<td>1350</td>
<td>690</td>
<td>570</td>
<td>280</td>
<td>19</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>HC 1</td>
<td>M/42</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a. (1999)</td>
<td>2350</td>
<td>1410</td>
<td>1090</td>
<td>310</td>
<td>12</td>
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<td>1.8</td>
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<td>HC 2</td>
<td>F/50</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a. (2000)</td>
<td>2420</td>
<td>1573</td>
<td>962</td>
<td>290</td>
<td>13</td>
<td>2.8</td>
<td>1.7</td>
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</table>

1. MG, Myasthenia Gravis, A, Anemia, PRCA, Pure Red Cell Aplasia;
2. Year of thymectomy;
3. Year of Bone Marrow sampling;
4. Peripheral Blood Lymphocyte count number (cells/mm3);
5. normal values: IgG 7-16 g/L; IgA 0.7-4.1 g/L; IgM 0.4-2.3 g/L.
6. Hys, Hystiocytosis; SLE, Systemic Lupus Erythematosus, MD, Myelodisplasia, HC, Healthy Controls.
Figure legends

Fig 1. T-Cell Receptor diversity of bone marrow-derived lymphocytes in thymoma patients. Panel A shows the CDR3 heterogeneity length analysis of Beta Variable 8 (BV8) families of T-cells isolated from peripheral blood and bone marrow (PB and BM, respectively) of five patients with thymoma and B lymphopenia. In the cases of patients 091, 147 and 157 we refer to CD8+ T cells. 051 and 009 profiles have been generated from unfractionated T cells. Panel B reports direct sequencing data of the major band found in BV8 families of patients 009, 051, 091, 147, 157. Panel C shows BV 8 profiles of two patients with thymoma and normal B cell counts (TC 011 and TC 087, respectively), three patient with thymoma-unrelated diseases (Hys 104, SLE 354, and MD27), and two healthy donors (HC 1 and HC 2, respectively). All profiles in panel C refer to CD8+ T cells, except for TC 011, Hys 104 and HC 1, in which data were generated on unsorted T cells. PCR products were separated on DNA sequencing polyacrylamide gel by using an automated ABI PRISM 377 apparatus (Perkin Elmer). Band intensity and size were evaluated with the Gene Scan software (Perkin Elmer) and expressed as relative fluorescence units and base pairs (rfu and bp, respectively). Panel D shows a synopsis of TCR repertoire expressed in bone marrow-derived CD8+ and CD8- T cell subsets. Open, grid and filled boxes represent BV families showing three standard profiles: normal (gaussian distribution); altered (less than five peaks with a not-gaussian distribution); and mono-oligoclonal profile (less than three major peaks), respectively. The absence of boxes represents undetectable BV families.
B

<table>
<thead>
<tr>
<th>UPN</th>
<th>BV8</th>
<th>CDR3</th>
<th>BJ</th>
<th></th>
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<tr>
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<td>CAS</td>
<td>SFGDGVN QPOH</td>
<td>FGDGTRLTVT</td>
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<tr>
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<td>FGNGLTRTVT</td>
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<tr>
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<td>FGPGLTRTVT</td>
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<tr>
<td>147</td>
<td>CAS</td>
<td>SLGVGAN QPOH</td>
<td>FGDGTRLSIL</td>
<td>1.5</td>
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<tr>
<td>157</td>
<td>CAS</td>
<td>SFG SGENVETQY</td>
<td>FGPGLTRTVT</td>
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1. BJ gene used.
<table>
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<th>% CD19+</th>
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<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>14</td>
<td>MD27</td>
</tr>
<tr>
<td>20</td>
<td>SLE 354</td>
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**Diagrams**

- **CD8**
  - + - + - + - + - + - +

  - BV Families:
    - 1
    - 2
    - 3
    - 4
    - 5.1
    - 5.3
    - 6.1
    - 6.2
    - 7
    - 8
    - 9
    - 12
    - 13
    - 14
    - 15
    - 17
    - 22
    - 24

- **UPN**
  - 091
  - 147
  - 157
  - TC087
  - HC2
  - MD27
  - SLE 354

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