Clonal Expansion of Lymphocytes Bearing the γδ T-Cell Receptor in a Patient With Large Granular Lymphocyte Disorder

By Henri Vie, Sylvie Chevalier, Richard Garand, Jean-Paul Moisan, Vincent Praloran, Marie-Claire Devilder, Jean-Francois Moreau, and Jean-Paul Souillou

Repeted analysis of peripheral blood lymphocytes (PBLs) from a patient with large granular lymphocytosis, neutropenia, and rheumatoid arthritis revealed that ~45% of PBLs displayed the following phenotype: CD3+, CD4-CD8+, CD16+, HNK-1+. This population was purified for further analysis by depletion with anti-CD4 and anti-CD8 monoclonal antibodies (MoAbs). The uniformity of the proliferating cells has been established by genetic analysis in some but not all cases. Most cases are characterized by proliferation of lymphocytes with a CD8+ phenotype together with the T cell receptor (TCR) α/β complex. The clonal nature of the proliferating cells has been established by genetic analysis in some but not all cases. However, a few cases have displayed unusual phenotypes. Three cases of CD3+, CD4−, CD8− granular lymphocytic proliferation have already been reported. Cells displaying a similar phenotype and bearing the TCR γδ have been described in rare normal lymphocytes and some acute leukemias. We report a clonal expansion of a CD3+, CD4+, CD8+, WT31+, TiyA+, TCRβ1−γ, δTC7−1 granular lymphocytic population associated with neutropenia and rheumatoid arthritis.

MATERIALS AND METHODS

Case report. B.M.S., a 56-year-old woman, was first admitted to our institution because of isolated asymptomatic neutropenia in January 1986. Physical examination revealed bilateral arthritis involving the joints of the hands, feet, and ankles. Hemoglobin (Hb) was 13.3 g/dL, WBC count 2.4 x 10⁹/L, polymorphonuclear neutrophil (PMN) count 0.2 x 10⁹/L, lymphocyte count 1.8 x 10⁹/L, and platelet count 200 x 10⁹/L. Abdominal ultrasonography revealed an enlarged spleen (maximum size 125 mm). BM aspirate was normocellular, with granulocytic maturation arrest at the metamyelocytic level and a moderate excess of lymphocytes. Because of the positivity of the rheumatoid factor (latex test 1/160, Waaler-Rose test 64 IU/mL), a diagnosis of rheumatoid arthritis with Felty syndrome was first proposed. The patient then received corticosteroid therapy which caused good response for the arthritis but had little effect on the neutropenia. After a steady-state period in November 1987, her hemogram showed a significant increase of lymphocytes (lymphocyte count 4.0 x 10⁹/L), which were large and contained azurophilic granules (large granular lymphocytes, LGLs) in 60% of cells. The PMN count was still low (0.35 x 10⁹/L). BM aspirate showed 47% lymphocytic infiltrate. Cytogenetic analysis of phytohemagglutinin (PHA)-cultured PBLs did not reveal any karyotypic abnormalities. At that time, the diagnosis of LGLD was reached.

Cell preparation and immunophenotyping. PB mononuclear cells were obtained by Ficoll-Hypaque density-gradient centrifugation. Immunophenotyping was performed on fresh, cryopreserved, and cultured cells. Surface markers were determined with a panel of monoclonal antibodies (MoAbs) by an indirect fluorescence technique and analyzed on a flow cytometer (ODAM ATC 3000, Wissembourg, France).

Monoclonal reagents. The different cell populations were studied with a panel of MoAbs classified according to the Oxford Workshop. OKT6 (CD1/thymocytes) was purchased from Ortho Diagnostic (Paris); T11 (CD2/sheep erythrocyte receptor), and T1 (CD5/pan-T cells) from Coultronics, Paris; IOT3 (CD3/mature T cells), IOT4 (CD4/help-inducer T cells), IOT7 (CD7/pan-T), IOT8 (CD8/suppressor/cytotoxic T cells), IOT14 (CD25/interleukin-2 receptor), IOT2a (HLA-DR), IOT5 (CD10/common acute lymphoblastic leukemia antigen (CALLA)), and IOT1 (CD3/mature B cells) from Immunotech, (Marseille, France); and Leu-7 [HNK1/subset of natural killer (NK) cells] and Leu-11b (CD16/low-affinity receptor for FcIgG) from Becton Dickinson, (Grenoble, France). WT31, which recognizes a framework determinant on the TCR α/β, was provided by Dr W. Tax (Amsterdam); TiyA, which recognizes the peptide encoded by the Vγ9 gene by Dr T. Hercend (Villejuif, France); TCRβ1 (anti-δγ) was produced by M. Brenner (D.F.C.I. Boston); and δTC7−1 (anti-β) by S. Hup from T Cell Science (Cambridge, MA).

Depletion of the CD4+/CD8+ T-cell population. Anti-CD4 (BL411, IgG2a) and anti-CD8 (B9.7, IgG2a) were provided by Dr D. Olive of INSERM U119 (Marseille Luminy, France). Cells were incubated at 10⁷/mL during 45 minutes on ice in 1 x 10⁷/mL interleukin-2 (IL-2), further incubated at 37°C for 45 minutes. The dead suspension was then washed and the suspension was further incubated at 37°C for 45 minutes. The dead cells were separated from the CD4−/CD8− cells on a Ficoll gradient. The final population is called CD4−/CD8− T cells.

Culture and enrichment in CD4+/CD8− T cells. PBLs were stimulated for 48 hours with PHA and then cultured with recombinant IL-2 (rIL-2 from Roussel-Uclaf Laboratory, France). After 4 days, the cells were purified by a CD8 magnetic bead enrichment kit (Miltenyi Biotec), and the CD4+/CD8− T cells were expanded with IL-2 until a sufficient number were obtained.

Clonal analysis. The clonal nature of the CD4+/CD8− T cells was confirmed by Southern blot analysis. After RNA isolation, cDNA was synthesized with random hexamers and reverse transcriptase, and amplified by PCR using TCRβ primers. The amplified products were fractionated on agarose gel and transferred to nylon membranes. Hybridization with TCRβ probes was performed, and autoradiography was used for detection. The results showed that the CD4+/CD8− T cells were clonally expanded.

DISCUSSION

The observation of a clonal expansion of γδ T cells in B.M.S. suggests that this patient may have a monoclonal disorder. The presence of a γδ T-cell clone in the peripheral blood suggests that the γδ T cells may play a role in the pathogenesis of the disease. The presence of a clonal γδ T-cell population in the peripheral blood of B.M.S. supports the hypothesis that γδ T cells may be involved in the pathogenesis of LGLD.

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weeks of culture, T cells were 89% positive with WT31 MoAb, showing that the WT31+ population had overgrown the WT31− population. This population is called CD4+CD8− T cells.

Cloning procedure for CD4+CD8−. CD4+CD8− cells were cloned under limiting conditions in 96-well U-bottom microtiter plates with 50,000 autologous lymphocytes, 5,000 allogeneic Epstein-Barr virus (EBV)-transformed B cells (both irradiated with 3,000 rad) and 150 U/mL rIL-2 (Roussel-Uclaf) in a final volume of 100 μL. Only microcultures with a chance of monoclonality >97% were considered clones. Subsequent expansion of the clones was performed by plating the cells between 1,000 and 5,000 per well in the culture condition described above.

Southern blot. Total genomic DNA were extracted from T cells as described by Grunenbaum et al.,14 and digested to completion with restriction endonucleases according to suppliers' indications (Amersham, UK and Boehringer-Mannheim, FRG). After electrophoresis of 10 μg digested DNA on 1% agarose gels, blotting onto Gene Screen Plus filter according to the supplier's indications. Hybridization with Dupont exposure to Kodak X Omat film for one or two days 15 mmol/L sodium citrate) and 0.1% SDS and sodium chloride, 100 L. Only microcultures of cloned under limiting conditions in 96-well U-bottom microtiter agarose gel and blotted on a cyanate method, was electrophoresed in a formaldehyde denaturing agarose gel and blotted on a Gene Screen Plus filter according to the supplier's indications. Hybridization was performed as described above for Southern blot.

Probes. The J probe PH60, containing the 700-base pair (bp) EcoRI-HindIII fragment from M13H60 subcloned in pUC9, includes the J1 segment.17 Because the two Jy1 and Jy2 regions are highly homologous,18 this probe detects rearrangements on both loci. Moreover, it allows assignment of the Vγ9, Jγy, and Cγy genes used in each rearrangement on analyzing restriction patterns with BamHI, EcoRI, HindIII, and KpnI digests19,20 (on a KpnI digest the PH60 probe detects a 1.8-kilobase (kb) band when a Vγ gene of the Vγ9 or Vγ9III gene families rearranges with the Jγy or Jy2 segment). Rearrangement between Vγ9 and JγP, shown in Fig 1, is characterized by a 12-kb band.19 Probe K20pR is a 1-kb-long genomic fragment containing the Vγ9 gene, which is the only V gene of the Vγ11 gene family.20 Probe IB10BB1 corresponds to the constant gene Cβ1 and is very homologous to the Cβ2 gene.21 Cβ1 and Cβ2 gene rearrangements are respectively detected on EcoRI and HindIII digests.21 Thus, all rearrangements of the locus can be detected, although this probe does not give any information about the V4, Df, and Jγ genes involved in the rearrangement.

Cytotoxic assay. A standard microtiter 3H release assay was used to measure lytic activity of effector cells against K562, an erythroleukemic cell line, and U937, a monocytic leukemic cell line. Autologous three-day PHA-activated lymphocytes were used as a negative control: Target cells were labeled by incubation at 37°C in 5 mL culture medium [RPMI + 10% fetal calf serum (FCS)] at 1 million per milliliter for 18 hours (overnight) with 100 μCi Na2 35CrO4 added. Target cells were washed three times, resuspended in complete medium, and adjusted at 30,000/mL. Effector cells were harvested and washed once, then resuspended at the indicated concentration; 100 μL suspension was transferred in triplicate to U-bottom microplate wells. Radiolabeled target cells in 100 μL were then added, and the microplates were incubated at 37°C for four hours. Supernatants were harvested with a multichannel pipette and counted in a γ-counter to determine the amount of 35Cr release. The percentage of cytosis was calculated according to the formula: % cytosis = ((EXP-SR)/(T-SR)) × 100, where the release of label from target cells without effector cells was defined as spontaneous release (SR), total (T) label released was determined by addition of a 1% cethanol solution, and experimental release (EXP) was the label released from targets with effector cells added.

BM cell fractionation and culture. The patient's BM cell aspirate was diluted 1:10 in Iscove's modified Dulbecco's medium

Table 1. Immunophenotyping of Patient's PBLs or CD4−CD8+

<table>
<thead>
<tr>
<th>MoAb</th>
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</tr>
<tr>
<td>Mo1</td>
<td>7</td>
<td>13</td>
</tr>
</tbody>
</table>

*Positive cells for each of the MoAb stainings.
(IMDM, GIBCO, Grand Island, NY) and separated on Ficoll-
Hypaque. The mononuclear cell fraction was washed twice in
IMDM-10% FCS and resuspended in the same medium: 10^6 mono-
nuclear BM cells were mixed with 10^4, 10^5, or 10^6 (CD4^+CD8^-) or
unselected PBLs in 5-mL sterile-cap polystyrene tubes. The cell
mixtures were gently pelleted by centrifugation (200 g for five
minutes) and incubated overnight in a 1-mL final volume in a
humidified atmosphere (37°C, 5% CO2). The cell mixtures were
then resuspended and plated in triplicate in collagen gels at 10^6 BM
cells per plate22 for colony assay according to a procedure previously
described. Recombinant GM-CSF (Genetic Institute, Boston) was
added to the culture medium at a final concentration of 100 U/mL.
Cultures were maintained for 14 days in a 5% CO2, 7% O2
humidified atmosphere before harvesting, fixation, and staining of
the gel for colony counting and morphologic analysis.

RESULTS

Immunophenotypic analysis. As shown in Table I, marker analysis of the patient's PBLs with MoAbs directed
against lymphocyte differentiation antigens revealed a dis-
crepancy between the number of CD3^+ cells and the total
number of CD4^+ and CD8^+ T cells (CD4^+ and
CD8^+ = 46%, CD3^+ = 93%). Similarly, we observed that
50% of the CD3^+ cells were WT31^- . On the other hand, both
the absolute number of CD4^+ and CD8^+ T cells was in the
normal range (data not shown), suggesting the presence of an
“added” unusual CD3^+ CD4^- CD8^- population.

To characterize this population further, we performed
complement-dependent cytolysis of CD4^+ and CD8^+ T cells.
Table I shows that the resulting CD4^-CD8^- population
expressed the CD3^+WT31^- CD16^- phenotype. Moreover,
this population reacted with TiγA, a MoAb which recognizes
the Vγ9 peptide, and TCRδ1, an anti-pan TCRδ. In contrast
those cells did not react with δTCS1, another anti-δ TCR
This Ti A^- population did not have an “activated” phenotype
since it surface expressed neither CD25 nor HLA-DR anti-
gen, the remaining DR^+ cells probably represented macro-
phages copurified during the depletion procedure, since 13%
of the CD4^-CD8^- were Mol^- .

Analysis of TCR gene rearrangements and expres-
sion. DNA from CD4^+CD8^- WT31^- and from fresh PBLs
were digested with BamHI, EcoRI, HindIII, and KpnI. To
define the genomic organization of the T cell rearranging γ
genes encoding for the TCR of those cells, we used the J-y1
probe pH6O. On KpnI digest, two rearranged bands were
detected on PBLs at 12 kb and 1.8 kb (Fig 2). The 1.8-kb
band showed rearrangement involving genes from the Vγ1
family (Fig 1 and Materials and Methods section). The
12-kb band showed the Vγ9-JγP rearrangement. The 1.8 kb
band, present on the PBL digest (as expected of a polyclonal

![Fig 2. γ T gene rearrangement analysis in patient's PBLs or
CD4^-CD8^-purified mononuclear cells. DNAs were extracted from this
cell population, digested with KpnI restriction enzyme, and hybridized to
pH60, a Jγ1 probe. The 1.8-kb band is absent on the CD4^-CD8^- digest.](image)

![Fig 3. Rearrangements of the T-cell rearranging γ gene (TRG)
in TiγA^- and TiγA^- clones from the patient. Germline bands at 16
kb and 9 kb are shown in the control lane (C). These germline
bands were still present in all five TiγA^- clones tested (G1 through
G6).](image)
Fig 4. Cytolytic activity of patient's PBLs or CD4^+ CD8^- against K562 or U937 or autologous PHA blast (Auto) performed before (a) and after (b) culture with rIL-2 (150 BRMP units/mL).
population) is absent on the T4–T8– digest (Fig 2), suggesting that these cells use only the Vγ9-JγP rearrangement. Usage of this rearrangement was confirmed by a Northern blot performed on total RNA extracted from the CD4–CD8– enriched population and hybridized to the pK20Pr (Vγ9 gene) probe (data not shown). With EcoRI and HindIII bands were in germline configuration (data not shown), as has also been observed for rearrangements involving JP1, JP, and JP2 segments. To address the question of the monoclonality of the TiγA+ population, we derived clones from CD4−CD8− purified cells. Thirty-four clones were analyzed for phenotype: four were WT31+ and CD4+ (thus representing the few which escaped depletion), three were WT31−TiγA−, and the remaining 27 were WT31+TiγA+. Figure 3 shows rearrangements observed (using KpnI digest and hybridization with PH60) for two WT31+ clones (WT1 and WT2), a WT31−TiγA+ clone (G10), and five of the TiγA+ clones. As expected, all five TiγA− clones displaying the 12-kb band showed the Vγ9-JγP rearrangement. For all clones tested, the germline bands were still present, indicating that those cells had rearranged the γ gene on only one of their chromosomes; thus it is very unlikely that they derived from a polyclonal population. For all WT31− clones, β genes were in germline configuration (data not shown).

Cytotoxicity. Cytotoxic activity of unseparated PBLs and the CD4−CD8− WT31− population was tested against autologous PHA blast, K562, and U937 (Fig 4a and b). Uncultured CD4−CD8− were negative (<10% at an effector/target (E/T) ratio of 50:1) on all three targets. Twenty-eight percent of cytotoxic activity was observed against U937 for unseparated PBLs (Fig 4a). In contrast, after six-day culture with IL-2, both PBLs and CD4−CD8− were cytotoxic against K562 and U937 (Fig 4b). As for uncultured cells, neither cultured PBLs nor cultured CD4−CD8− were cytotoxic against autologous PHA blasts (Fig 4a and 4b).

Effect on in vitro BM CFU-GM growth. A CFU-GM colony formation assay was performed after overnight preincubation of the autologous BM mononuclear cell fraction and either separated CD4−CD8− WT31− or unselected PBLs. No significant differences were observed between the effect of either CD4−CD8− WT31− or unseparated PBLs on CFU-GM colony formation (Table 2).

| Table 2. Effect of BM Mononuclear Cell Preincubation in the Presence of Either Unseparated PBLs or CD4−CD8− on the Percentage of CFU-G, CFU-M, and CFU-GM |
|-----------------|-----------------|-----------------|
| Preincubation (18 h) | 10^6 BM Mononuclear Cells + Medium Alone (%) | 10^6 BM Mononuclear Cells + 10^6 PBLs (%) | 10^6 BM Mononuclear Cells + 10^6 CD4−CD8− (%) |
| CFU-G           | 36 ± 3          | 34 ± 5          | 41 ± 7          |
| CFU-M           | 52 ± 4          | 51 ± 4          | 51 ± 2          |
| CFU-GM          | 12 ± 5          | 15 ± 3          | 8 ± 5           |

Mononuclear cell fractions from the patient’s BM aspirate were incubated overnight in the conditions shown and then plated for colony formation assay as described in the Materials and Methods section.
penia results from a mechanism that does not implicate the leukemic LGL population directly or that their target may not always be the granulocyte at the progenitor stage. Further study is needed to specify the relationships, if any, between the mature neutrophil population and this particular LGL subset.

REFERENCES


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REFERENCES


Clonal expansion of lymphocytes bearing the gamma delta T-cell receptor in a patient with large granular lymphocyte disorder

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