Clonal B-Cell Populations in Patients With Idiopathic Thrombocytopenic Purpura

By D. van der Harst, D. de Jong, J. Limpens, Ph. M. Kluin, Y. Rozier, G.J.B. van Ommen, and A. Brand

Idiopathic thrombocytopenic purpura (ITP) may be associated with other autoimmune diseases and the development of lymphoproliferative malignancies. In Sjögren’s disease, Graves’ disease, and essential mixed cryoglobulinemia, which are also associated with the development of B-cell neoplasia, clonal B-cell expansions have been detected. Eleven patients with ITP were investigated for the presence of a clonal excess (CE) using \( x \)-\( \lambda \) flow cytometry and DNA analysis for rearrangement of immunoglobulin heavy and light chain genes in blood and/or spleen lymphocytes. In 10 of 11 patients, clonal B-cell populations were found by one or both tests. In three of these patients, oligoclonal B-cell populations were suggested by the combined findings. In all four patients with a small paraproteinemia, the isotype was confirmed by either flow cytometry or DNA rearrangement analysis. Our data suggest that the oligoclonal expansions are not restricted to CD5+ B cells, as in the majority of patients this subset was below the detection level of flow cytometry or DNA rearrangement analysis. None of the patients developed clinical manifestations of malignant lymphoma during a follow-up period of 10 to 44 months after sampling. We conclude that clonal excess populations of B cells are not a unique feature of malignant lymphoma, but may occur in autoimmune diseases, suggesting a benign (oligo)clonal B-cell proliferation.

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MATERIALS AND METHODS

Patients. Eleven patients (6 women, 5 men) ranging in age from 21 to 70 years, with ITP and platelet counts below 50 \( \times \) 10^9/L, were tested. The interval between diagnosis of ITP and CE testing ranged from 3 months to 18 years.

Seven patients had presented with acute onset ITP, were refractory for corticosteroids; six of them subsequently were splenectomized, of whom three remained in complete remission and three patients showed a relapse. One patient presented with corticosteroid-resistant ITP during pregnancy but came into spontaneous remission 6 months after delivery. At the moment of analysis, four patients received corticosteroids, and three patients were tested before therapy.

Four patients had chronic ITP, refractory for corticosteroids, splenectomy, vincristine, and alkylating immunosuppressive drugs. The spleen of one patient was removed 10 years before the actual investigation and could not be tested. At the moment of analysis, three patients were on prednisone therapy and one on androgen therapy.

All patients were tested at least once during the active phase of the disease, and two patients were tested in complete remission, off therapy. Peripheral blood samples of 24 healthy donors and spleen specimens of 25 cadaveric kidney donors served as controls for flow cytometry.

To exclude the possibility that transient clonal expansions may arise during recovery from drug therapy, the control group was extended with five patients who had previously received chemotherapy. Three patients were in complete remission from acute myelogenous leukemia, and the interval between testing of peripheral blood samples and the last course of chemotherapy was less than 6 weeks. Spleen samples of patients with autoimmune hemolytic anemia (one case, in remission) and thrombotic thrombocytopenic purpura (one case, in remission after corticosteroids, vincristine, and plasma exchange), were tested.

Preparation of leukocyte suspensions. Mononuclear cells from heparinized venous blood were isolated by Ficoll-Isopaque density centrifugation and extensively washed with Hanks’ balanced salt solution (GIBCO, Grand Island, NY) before cryopreservation in RPMI 1640 (GIBCO), supplemented with 10% selected human serum and 10% dimethyl sulfoxide.

After thawing, the cells were washed three times with Hanks’ and resuspended in RPMI 1640. Parts of spleens were minced, using the blunt end of a syringe plunger. The cells were washed with Hanks’ and cryopreserved as described. After thawing, the cells were washed three times with Hanks’ and resuspended in RPMI 1640.

Immunofluorescence. After thawing, 2 \( \times \) 10^5 cells were incubated with anti-\( \kappa \) and anti-\( \lambda \) monoclonal antibodies (MoAbs) (Becton Dickinson, Mountain View, CA) for 30 minutes at 4°C, washed,

From the Departments of Immunohaematology and Bloodbank, Human Genetics, Sylvius Laboratory, and Pathology, University Medical Centre, Leiden, The Netherlands.

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Address reprint requests to A. Brand, MD, University Medical Centre, Department of Immunohaematology and Bloodbank, Rijnburgerweg 10, Bldg 1, E3-Q, PO Box 9600, 2300 RC Leiden, The Netherlands.

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and incubated with goat antimouse/FITC for 30 minutes at 4°C. After washing, the cells were resuspended in 1% paraformaldehyde before analysis. Flow cytometric analysis was performed on a mercury-arc lamp equipped FACS Analyzer (Becton Dickinson), using a quantification technique described by Ligler et al.\(^6\) for the detection of a CE.

Duplicate experiments were performed on a laser-powered FACSscan (Becton Dickinson), using the Consort 30 software and Kolmogorov-Smirnov statistics.\(^2\) A difference value greater than 0.15 was considered to be significant for spleen as well for peripheral blood samples.\(^3\)

To determine the sensitivity of the CE test, clonal B cells from four patients with chronic lymphocytic leukemia (CLL) and anti-Leu 16 (CD20) MoAbs (Becton Dickinson) for 30 minutes at 4°C, washed, and screened for the presence of CD20+CD5+ cells on a FACScan.

**B-cell enrichment and cell sorting.** B cells from the spleen samples of patients 3, 4, 6, 10, and 11 were purified using standard sheep red blood cell rosetting techniques. The purity of the nonrosetting B-cell suspension was 90%. Spleen cells of patient 10 were incubated with conjugated anti-Leu 1 (CD5) and anti-Leu 16 (CD20) MoAbs (Becton Dickinson) for 30 minutes at 4°C, washed, and screened for the presence of CD20+CD5+ cells on a FACScan.

**DNA analysis.** High-molecular-weight DNA was isolated according to standard methods, as previously described.\(^1\) Briefly, DNA was digested to completion with restriction enzymes HindIII, BamHI, and EcoRI; size-fractioned over a 0.7% agarose gel; and analyzed according to standard methods, as previously described.\(^*\) The following probes were used: a 2.5-kb Sau3AI germline fragment of the Ig heavy chain joining region (JH), a 2.5-kb EcoRI germline fragment of the Ig k light chain constant region (Ck), and a 3.5-kb EcoRI-HindIII germline fragment of the Ig l light chain constant region (Cx). To determine the sensitivity of this method, serial dilutions of CLL cells in normal peripheral blood lymphocytes were subjected to Southern blot analysis.

**Serum investigation.** Serum samples of all patients were screened for the presence of monoclonal Igs using agar and immunoelectrophoresis, and immunofixation techniques as described by Van Nieuwkoop et al.\(^*\)

**RESULTS**

Flow cytometric analysis of 25 spleens from kidney donors and of peripheral blood samples from 24 healthy blood donors showed no clonal excess. In the control group of five patients no clonal excess could be detected after chemotheraphy and prednisone (D < .15), although the D value tended to be higher compared with normal donors. On Southern blot analysis, no rearranged bands could be demonstrated.

The sensitivity of the CE test was assessed by mixing healthy donor lymphocytes with clonal B cells from four different patients with CLL. Clonal CLL cells, 1%, 3%, 3%, and 10%, respectively, could be detected using flow cytometry. Serial dilutions of CLL cells in normal peripheral blood lymphocytes were subjected to Southern blot analysis. In accordance with the literature, a sensitivity limit of 5% clonal cells per sample was obtained.\(^13\)

The patients' results are summarized in Table 1, and k/\(\lambda\) fluorescence histograms from eight patients are shown in Fig.

<table>
<thead>
<tr>
<th>Patient</th>
<th>ITP</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Material</th>
<th>Flow Cytometry</th>
<th>DNA Rearrangement</th>
<th>Paraproteinemia</th>
<th>Previous Therapy</th>
<th>Follow-up</th>
</tr>
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<tr>
<td>1</td>
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<td>21</td>
<td>F</td>
<td>PBL</td>
<td>(\kappa)</td>
<td>JH - CK (\kappa)</td>
<td><strong>IgG-(\kappa)</strong></td>
<td>-</td>
<td>CR</td>
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<td>F</td>
<td>Spleen</td>
<td>(\lambda)</td>
<td>JH + CK (\kappa)</td>
<td><strong>IgG-(\kappa)</strong></td>
<td>ISD</td>
<td>CR</td>
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<tr>
<td>3</td>
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<td>48</td>
<td>M</td>
<td>PBL</td>
<td>(\lambda)</td>
<td>JH - CK (\kappa)</td>
<td><strong>IgG-(\kappa)</strong></td>
<td>ISD</td>
<td>CR</td>
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<tr>
<td>4</td>
<td>Acute</td>
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<td>M</td>
<td>Spleen</td>
<td>(\kappa)</td>
<td>JH - CK (\kappa)</td>
<td><strong>IgG-(\kappa)</strong></td>
<td>ISD</td>
<td>PT</td>
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<tr>
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<td>JH - CK (\kappa)</td>
<td><strong>IgG-(\kappa)</strong></td>
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<td>PT</td>
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<td>JH - CK (\kappa)</td>
<td><strong>IgG-(\kappa)</strong></td>
<td>ISD</td>
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<td>Spleen</td>
<td>(\kappa)</td>
<td>JH - CK (\kappa)</td>
<td><strong>IgG-(\kappa)</strong></td>
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<tr>
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<td>JH - CK (\kappa)</td>
<td><strong>IgG-(\kappa)</strong></td>
<td>ISD</td>
<td>PT</td>
</tr>
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</table>

**Table 1. Summary of Patient Data, and Results From Flow Cytometric and DNA Analysis**

Abbreviations: PBL, peripheral blood lymphocytes; NT, not tested; ISD, immunosuppressive drugs; PT, persistent thrombocytopenia.

*In complete remission, 4 months later than first sample.

†Intravenous γ-globulin.

‡In complete remission, off therapy.
1. In 10 of 11 patients, clonal B-cell populations could be detected using flow cytometry and Ig gene rearrangement analysis of the heavy and light chain genes. In seven cases, clonality was found by both techniques. In three cases, clonality was detected by either one or both techniques. Clonality was only detected in single blood or spleen suspensions by one or both techniques in several cases.

For instance, in patient 3 clonal expansions in peripheral blood were detected using flow cytometry. These clonal expansions could not be confirmed by Southern blot analysis. Conversely, a clonal expansion in the spleen was found using DNA rearrangement analysis that was not detected by flow cytometry (Table 1).

Three cases showed multiple clones and are of special interest (Fig 2). In patient 9, flow cytometry showed a $\kappa$-positive clonal excess, whereas DNA analysis showed a population with $\lambda$-gene rearrangement. Using immunofixation, two different serum paraproteins of IgM-$\kappa$ and IgM-$\lambda$ were seen. The results in patient 11 were largely similar to the results in patient 9, except for the presence of a $\kappa$ paraproteinemia only. In patient 10, flow cytometry indicated both $\kappa$ and $\lambda$ excess populations. Also, DNA rearrangement studies were highly suggestive of two clonal populations on the basis of distinct differences in hybridization intensity with both light chain probes.

In cases 3, 4, 6, 10, and 11, B cells were enriched and subsequently tested by Southern blot analysis. In all cases, intensification of the hybridization signal for the rearranged JH genes was observed after enrichment (Fig 3).

In view of the possibility that autoreactive B cells are
Fig 2. Southern blot analysis of three cases of ITP. Sizes are given in kilobase pairs (kb). Germline bands are indicated with an arrowhead, rearranged bands with a dash. A weakly hybridizing pseudogene of CA gives rise to a 4.8-kb fragment, which is indicated with an open arrowhead. One rearranged heavy chain allele is seen in case 10, both in EcoRI (lane a) and BamHI (lane b) digested DNA. A rearranged λ light chain allele and a rearranged λ light chain allele are detected in HindIII digested DNA and EcoRI digested DNA, respectively. Case 1 has a rearranged heavy chain allele (EcoRI) and a rearranged λ light chain allele (BamHI), while the λ genes are in germline configuration. Case 11 has a rearranged heavy chain allele (BamHI) and a rearranged λ light chain allele (EcoRI). The κ light chain genes are in germline configuration. It should be noted that a restriction fragment length polymorphism for EcoRI generates an 18-kb germline fragment, which is absent in case 1, on hybridization with the CA probe in cases 10 and 11.

restricted to the CD5⁺ subpopulation, we enumerated CD5⁺ and CD5⁻ B-cell subpopulations in splenic suspensions and performed one Southern blot analysis with enriched CD5⁺ B cells. The number of splenic CD5⁺ B cells in ITP patients was 3.4 ± 2.2% (n = 9; mean ± SD), compared with 5.0 ± 4.2% (n = 13) of mononuclear cells in normal controls.

Splenic cells from case 10 were sorted into CD20⁻CD5⁺ and CD20⁺CD5⁺ B cells, as depicted in Fig 4. The results of DNA analysis of CD5⁺ and CD5⁻ B cells in this case, compared with the native sample and unsorted enriched B-cell sample, are given in Fig 5. Apart from the germline bands, different rearranged bands are detected in the CD5⁺ and CD5⁻ samples, of which one is also seen in the native sample and two in the B-cell enriched sample.

None of the patients developed an NHL during 10 to 44 months (mean follow-up 15 months) after sampling, or during 9 months to 18 years after the initial diagnosis of ITP.

DISCUSSION

Using flow cytometric analysis, Ig gene rearrangement studies, and serum protein analysis, we demonstrated the presence of clonal B-cell populations in 10 of 11 patients with ITP. Patients 1 and 6 were tested during active disease as well as in remission, when clonal expansions had disappeared. In 3 of 10 cases, our data indicate an oligoclonal nature of these B cells.

In case 9, the presence of two different paraproteinemias was associated with a single clonal excess population detected by flow cytometry, and a different clonal B-cell population on Southern blot analysis. In patient 11, both flow cytometry and Southern blot analysis indicated the presence of two clonal populations associated with a single paraproteinemia. In case 10, both techniques indicated the presence of two B-cell clones, but no paraproteinemia was observed. Because the detection limit of both techniques is approximately 1% to 10% under the conditions used, smaller (oligo)clonal B-cell populations may have escaped detection.
by one or both techniques. Flow cytometric detection of B-cell clones depends on the number of cells, as well as the intensity of fluorescence. Detection of clones by Southern blot analysis depends only on the cell number of the clones, relative to the total number of cells. This may account for the finding of different B-cell clones on the basis of light chain expression in cases 9 and 11, and for differences between blood and spleen samples in individual patients.

The nature of clonal B-cell populations in patients with ITP is unknown. Literature data point to a possible role of CD5+ B cells in the recognition of self-antigens and the production of polyreactive low-affinity autoantibodies. Therefore, it might be postulated that the observed B-cell clones are CD5+ and also produce antiplatelet antibodies. However, such a direct involvement seems unlikely because the total number of CD5+ splenic B cells is equally low in ITP and normal controls, and in the majority of patients clonal populations within this subset may well be below the detection levels of flow cytometry and Southern blot analysis. Furthermore, Southern blot analysis of sorted CD5+ and CD5- splenic B cells in case 10 indicates that rearrangements are not confined to CD5+ B cells alone.

Therefore, it seems more likely that the presence of (multiple) B-cell clones and the production of antiplatelet antibodies in ITP may be the result of B-cell dysregulation, rather than that the clones are directly involved in the production of the antibodies.

In view of the relatively high incidence of clonal populations in our patient group and the general low risk of lymphoma development in ITP patients and the patient population discussed during follow-up, the possibility that the (oligo)clonal populations represent early or abortive neoplastic stages of lymphoma seems unlikely, but cannot be excluded.

The finding of clonal B-cell proliferations in autoimmune diseases is underscored by the uniform detection of a single light chain rearrangement in benign lymphoepithelial lesions in Sjögren's disease, and by immunocytochemical studies in Graves' disease and cryoglobulinemia. In view of these findings and our data in ITP, it is important to note that flow cytometric analysis (with or without sophisticated techniques as described by Nakano et al) and Ig gene rearrangement studies have to be interpreted cautiously, because neither can make an absolute distinction between malignant and benign clonal B-cell proliferations.
Fig 5. Southern blot analysis of case 10. Spleen cells were enriched for B cells using standard sheep red blood cell rosetting techniques. Splenic cells were sorted in CD5- and CD5+ fractions on a flow cytometer as described in Materials and Methods. All suspensions were digested with EcoRI and hybridized with a JH probe. Lane marked S contains the native sample (spleen); lane B contains the enriched B-cell suspension before cell sorting; and lanes CD5- contain B cells separated on the basis of CD5 expression. In the CD5+/− populations, apart from germline bands (arrowhead), different rearranged bands (−−) are detected, of which one is also seen in the native suspension and two in the B-cell enriched fraction.

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