Evidence for Peripheral Blood B Lymphocyte but Not T Lymphocyte Involvement in Multiple Myeloma

By James Berenson, Rex Wong, Kenneth Kim, Nathaniel Brown, and Alan Lichtenstein

Because there is controversy regarding whether subsets of peripheral blood lymphocytes (PBLs) are part of the malignant clone in patients with multiple myeloma, we studied this question by immunoglobulin and T cell receptor gene analysis. Southern blot analysis with antibody probes demonstrated clonal immunoglobulin gene rearrangements in PBLs of seven of nine patients that were identical to those seen in their marrow plasma cells. Circulating plasma cells were not detected in any of these patients. In contrast, no patient demonstrated clonally rearranged T cell receptor genes. In one sequentially studied patient, PBLs obtained at diagnosis when he had stage I (Durie-Salmon) contained only germine DNA, while analysis of PBLs at relapse (stage III) revealed a clonally rearranged band. These data confirm the notion that circulating lymphocytes in patients with myeloma are part of the malignant clone and, furthermore, these malignant cells are of B cell rather than T cell lineage.

MULTIPLE MYELOMA is regarded as a disease of immunoglobulin-producing cells. Although the predominant cell of this marrow-based neoplasm has the morphological characteristics of a plasma cell, data have accumulated that other lymphoid cell types are also part of the malignant process. In patients, a significant number of peripheral blood lymphocytes (PBLs) bear the unique paraprotein idiotype found in the plasma cells. These data would suggest that an earlier cell in B cell differentiation is part of the malignant clone. Moreover, some workers have shown that idiotype-bearing T cells also circulate in the peripheral blood of these patients.

On the other hand, King and Wells have demonstrated that the cytophilic IgG idiotype may remain bound to Fc receptors on PBLs even after extensive washing and incubation. These investigators failed to show any evidence of PBLs with an endogenous surface idiotype when using a rosette assay. Recently, two other groups have also found significant numbers of idiotype-bearing PBLs when using similar assays. Thus, there is controversy regarding whether PBLs are part of the malignant clone in multiple myeloma and, furthermore, which lymphocyte subsets are involved in the disease.

To address this controversy, we have searched for antibody and T cell receptor (TCR) gene rearrangements to determine whether significant numbers of clonal malignant B and/or T lymphocytes exist in the peripheral blood of patients. Gene rearrangements can be used to accomplish this goal because clonally derived B or T cell populations have unique rearrangements of antibody or TCR genes, respectively. This DNA analysis avoids the problems with passively adsorbed antibody which has confounded previous studies.

MATERIALS AND METHODS

Patients. Nine patients with multiple myeloma as defined by the Myeloma Task Force were studied. All patients underwent bone marrow examination, serum protein electrophoresis, immunoelctrophoresis, bone surveys, routine chemistries, CBCs, and 24-hour urine collection for determination of creatinine and protein. Patients were staged according to the Durie-Salmon classification.

Isolation of peripheral blood lymphocytes, granulocytes and bone marrow plasma cells. Peripheral venous blood and bone marrow samples were obtained from all nine patients after informed consent and in accordance with the Human Subjects Review Boards of the Veteran’s Administration Wadsworth and UCLA Medical Center. Peripheral blood was centrifuged to separate the cells from the serum. The cells were exposed to Histopaque (Pharmacia Fine Chemicals, Piscataway, NJ) 1077 and 1119 density gradient separations yielding enriched populations of plasma cells. Southern blot analysis. Genomic DNA was prepared from PBLs, granulocytes, and bone marrow plasma cells from each patient according to the method of Perry et al. The DNA was digested with restriction endonucleases BamHI, EcoRI or HindIII (Bethesda Research Laboratories, MD). Ten micrograms of digested DNA was electrophoresed in 0.6% agarose gels at 1 to 2 V/cm for 42 hours and transferred to nylon membranes by the method of Southern. Parallel lanes of similarly digested granulocyte, PBL, and plasma cell DNA samples from each patient were electrophoresed on the same agarose gel.

Recombinant probes for the human heavy chain immunoglobulin (Ig) gene segments Jκ and Cκ were donated by L. Hood et al (California Institute of Technology, Pasadena, CA) and were subcloned in pBR322. The Jκ probe is a 3.2-kilobase pair (kb), human genomic fragment extending from JH2 to the HindIII site in the Lκ-Cκ intervening sequence. The Cκ probe is a 2-kb genomic clone containing the complete sequence of Cκ; this probe hybridizes with all four Cκ-subclass gene fragments, which are reported to be ≥90%
homologous. The $C_{\lambda}$ probe is a 4.2-kb BamHI-XhoI human genomic fragment that contains the $C_{\lambda}$ gene subclone in pBR322 and was subcloned from a Charon 28 phage that was donated by J. Ravetch et al. (Harvard University, Cambridge, MA). Recombinant probes for the human light chain Ig gene segments $C_{\lambda}$ and $J_{\lambda}$, were donated by P. Leder. The $C_{\lambda}$ probe is a 5.0-kb EcoRI-HindIII human genomic fragment that is also subcloned in pBR322. The $J_{\lambda}$-C, probe is a 2.5-kb EcoRI fragment in pBR325. The TCR gene probe is a $\beta$-TCR cDNA clone called JUR-beta 2, subcloned in pBR322 and donated by Dr. T. Mak (University of Toronto).

Probes DNAs were labeled with $[^{32}P]dCTP$ (deoxycytidine triphosphate) by nick translation. Hybridization and washing were essentially those described by Wahl et al. After washing, the hybridized blots were autoradiographed using Kodak XAR-5 film and intensifying screens at -70°C for four to 14 days.

RESULTS

All patients were male and ranged from 53 to 79 years of age. Six patients had IgGk myeloma, two had IgA\lambda myeloma, and one had IgG\lambda myeloma (Table 1). Three patients were in stage IIA; three, IIIB; two, IIIA; and one, IA at diagnosis and IIIB at relapse (patient no. 6, Table 1). No patient had obvious circulating plasma cells on microscopic examination of their peripheral blood.

The results of Southern blot analysis with antibody and $\beta$-TCR gene probes are summarized in Table 1. Seven of nine patients showed clonal antibody gene rearrangements in PBLs identical to those seen in their respective marrow plasma cells. In all six patients with IgGk myeloma, this was demonstrated by identification of rearranged light-chain genes and/or heavy-chain genes. In one of two IgA patients, a rearranged light-chain band was detected, whereas only germline DNA was found in the other. In the one patient with IgG\lambda myeloma, hybridization with the $J_{\lambda}$ probe only revealed germline DNA.

The two patients with only germline DNA in their PBLs (patients no. 8 and 9), had clearly detectable rearranged Ig bands in their marrow plasma cells. Clinically, they had progressive symptomatic myeloma and were in Durie-Salmon stage IIA and IIIA. The seven patients with clonally rearranged Ig genes in PBLs were in stage IIA (two patients), IIIA (one patient), and IIIB (four patients). Thus, there was no correlation between clinical stage and DNA analysis in this small patient sample. However, one patient, (patient no. 6) did not show a new rearranged band in PBLs at the time of diagnosis when he was in stage IA, but at relapse when he was in stage IIIB, his PBLs contained clonally rearranged DNA (Fig 1). The germline $J_\mu$ band is approximately 18-kb, and narrow plasma cells, at diagnosis and relapse, show a rearranged band at 13 kb. This rearranged band is only present in PBLs at relapse (Fig 1).

No patient demonstrated clonal $\beta$-TCR gene rearrangements in the PBL or plasma cell preparations.

DISCUSSION

The aim of the present study was to investigate whether significant numbers of peripheral blood lymphocytes are part of the malignant clone in multiple myeloma. The detection of clonal Ig and TCR gene rearrangements has been used to demonstrate the clonal origin of other B and T cell–derived tumors. We have used these same gene probes to demonstrate that clonal antibody gene rearrangements but not TCR gene rearrangements occur in the PBLs of most patients with multiple myeloma. These results indicate that peripheral blood B lymphocytes but not T lymphocytes are part of the malignant clone in multiple myeloma.

These data confirm the work of other investigators who demonstrated peripheral blood monoclonal B cells by either abnormal surface membrane $k/\lambda$ light-chain ratios or positive surface immunofluorescence with antiidiotypic reagents. However, these results contradict those of Pilarski et al, King and Wells, and Gobbi et al who failed to find monoclonal B lymphocytes in the peripheral blood of myeloma patients. Pilarski et al studied only three patients in remission and used antiidiotypic reagents. In our study, we only studied patients with active disease. It is interesting to note that the patient with the lowest tumor burden at diagnosis (patient no. 6) only demonstrated involvement in the peripheral blood after the development of clinical relapse with a marked increase in tumor burden. King and Wells studied two patients with active myeloma but with fairly small serum M spikes and two patients in clinical remission.

Table 1. Clinical Characteristics and Southern Blot Analysis

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Isotype</th>
<th>Light Chain</th>
<th>Constant Region</th>
<th>$J_{\mu}$</th>
<th>$\beta$-TCR Genes in PBLs and Plasma Cells</th>
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<tbody>
<tr>
<td>1</td>
<td>Gk</td>
<td>R</td>
<td>R</td>
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<td>ND</td>
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<tr>
<td>2</td>
<td>Gk</td>
<td>ND</td>
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<td>R</td>
<td>G</td>
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<td>Gk</td>
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<td>6</td>
<td>Gk</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
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<tr>
<td>At diagnosis</td>
<td>A\lambda</td>
<td>ND</td>
<td>ND</td>
<td>R</td>
<td>ND</td>
</tr>
<tr>
<td>At relapse</td>
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<td>9*</td>
<td>A\lambda</td>
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</table>

Abbreviations: R, rearranged DNA identical to bone marrow plasma cells; G, germline or unrearranged DNA; ND, not done.

*Both these patients demonstrated antibody gene rearrangements in marrow plasma cells.
These investigators used a rosette assay to detect idiotype-bearing cells after vigorous washing and failed to demonstrate PBLs bearing an endogenous idiotype when using these techniques. This negative result may also reflect the low tumor burden of their patients and the insensitivity of this assay. Gobbi et al. used pokeweed mitogen-stimulated peripheral blood lymphocytes from multiple myeloma patients to demonstrate the polyclonality of these cells by using k/\lambda ratios. However, a small but significant number of monoclonal cells may not be detected by this technique. It is also possible that the malignant PBLs may not respond to mitogenic stimulation. In contrast to the aforementioned techniques, Southern blot analysis is exceptionally sensitive. Most investigators estimate that as low as a 1% involvement of a cell mixture can be identified by this technique.

In our study, we have not shown clonal TCR gene rearrangements in any of our patients. These results would suggest that clonal T cells do not exist in multiple myeloma as has been reported by other workers. However, these investigators used antidiotype-specific antisera to demonstrate T cell involvement in a single patient with myeloma. It is well known that there is marked expansion of T cells that bear Fc receptors for the idiotype, and these "clonal" T cells were probably T cells with idiotype bound to their Fc receptors.

The common acute lymphoblastic leukemia antigen (CALLA) has been demonstrated on the surface of bone marrow cells in patients with myeloma. Because CALLA is normally thought to be present on B lymphoid cells during early stages of differentiation, these data suggest an oncogenic event in myeloma occurring in similar early precursor cells. The presence of pre-B lymphoid cells that express homologous idiotype in two patients with IgA myeloma also supports this notion. We are presently using antibody gene analysis to determine whether earlier stages in B cell differentiation have clonal expansion of cells.

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