In Vitro Tests That Predict Tumor-Associated Idiotype Levels in the Serum of Patients With B Cell Lymphomas and Leukemias

By Richard A. Miller, James N. Lowder, Julie Gralow, Timothy Meeker, and Ronald Levy

The presence of circulating tumor idiotype interferes with the in vivo effectiveness of anti-idiotype antibodies. We developed two assays that permit identification of patients with high levels of serum idiotype without the need for first producing an anti-idiotype antibody. A cell suspension made from the tumor was cultured for seven days with or without phytohemagglutinin (PHA) and/or phorbol myristate acetate (PMA). Ig secretion in vitro by patients' tumor cells varied. In 4 patients, no secretion in vitro occurred, 5 patients had low levels, and 5 patients had high levels of Ig secretion. In three patients, Ig secretion occurred only after stimulation with PHA, PMA, or both. Spontaneous or induced immunoglobulin secretion in vitro is related to the levels of tumor idiotype secretion that exist in vivo. Eight patients with serum idiotype levels >100 µg/mL (mean 265 µg/mL), had a minimum of 1.0 µg/10^6 cells of idiotype secretion in vitro. Nine patients with serum idiotype levels <30 µg/mL (mean 3.7 µg/mL), had <0.5 µg/10^6 cells of idiotype secretion in vitro. In another assay, the levels of IgM and IgM λ in patients' sera were compared with those in normal serum. An imbalance in the relative amounts of IgM and IgM λ indicated high levels of circulating idiotype in the serum, but this assay was less sensitive than the in vitro secretion assay and limited to IgM-secreting tumors. These assays will be useful for future clinical studies using anti-idiotype antibodies.

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Table 1. Characteristics of Patients' Tissues

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis*</th>
<th>Tissue Source*</th>
<th>Ig Type</th>
<th>Percentage of Cells†</th>
<th>Tumor</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.B.</td>
<td>NLPD</td>
<td>Spleen</td>
<td>μ, κ</td>
<td>94</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>R.B.</td>
<td>NLPD</td>
<td>LN</td>
<td>μ, κ</td>
<td>53</td>
<td>45</td>
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</tr>
<tr>
<td>J.C.</td>
<td>NLPD</td>
<td>Spleen</td>
<td>γ, λ</td>
<td>86</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>K.C.</td>
<td>NLPD</td>
<td>LN</td>
<td>γ, λ</td>
<td>84</td>
<td>16</td>
<td></td>
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<tr>
<td>N.D.</td>
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<td>μ, λ</td>
<td>74</td>
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<td>P.E.</td>
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<td>LN</td>
<td>μ, κ</td>
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<td>T.G.</td>
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<td>PBL</td>
<td>μ, γ, λ</td>
<td>93</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>C.G.</td>
<td>NM</td>
<td>Spleen</td>
<td>μ, κ</td>
<td>79</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>D.H.</td>
<td>CLL</td>
<td>PBL</td>
<td>μ, κ</td>
<td>86</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>B.J.</td>
<td>NLPD</td>
<td>LN</td>
<td>μ, κ</td>
<td>69</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>C.J.</td>
<td>NLPD</td>
<td>LN</td>
<td>μ, κ</td>
<td>86</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>B.L.</td>
<td>NLPD</td>
<td>LN</td>
<td>μ, κ</td>
<td>84</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>C.P.</td>
<td>NM</td>
<td>LN</td>
<td>μ, λ</td>
<td>73</td>
<td>25</td>
<td></td>
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<tr>
<td>R.P.</td>
<td>CLL</td>
<td>PBL</td>
<td>μ, κ</td>
<td>79</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>L.R.</td>
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<td>μ, λ</td>
<td>73</td>
<td>24</td>
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<td>D.S.</td>
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<td>μ, λ</td>
<td>85</td>
<td>13</td>
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<td>PBL</td>
<td>μ, κ</td>
<td>94</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>M.V.</td>
<td>DLDP</td>
<td>PBL</td>
<td>μ, κ</td>
<td>84</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

* NLPD, nodular lymphoma, lymphocytic, poorly differentiated type (follicular, small cleaved cell); DM, diffuse lymphoma, mixed histiocytic-lymphocytic type (diffuse mixed small cleaved and large cell); DLDP, diffuse lymphoma, lymphocytic, poorly differentiated type (diffuse, small cleaved cell); NM, nodular lymphoma, mixed histiocytic-lymphocytic type (follicular, small cleaved and large cell); CLL, chronic lymphocytic leukemia; PLL, prolymphocytic lymphoma; PBL, peripheral blood lymphocytes.
† Percentage of tumor cells and T cells determined by immunofluorescence staining and analysis using flow cytometry. Calculation of tumor cells based on staining with either anti-κ or anti-λ reagent. Percentage of T cells was determined using anti-Leu-4.

placer in each of four wells in a 24-well tissue culture plate (Costar, Cambridge, MA). Either 1.0 μg of pyrohemagglutinin (PHA, Burroughs Wellcome, Research Triangle, NC) or 10 ng of phorbol myristic acetate (PMA, Sigma, St Louis) or a combination of both at the same final concentrations were added to individual wells. Control and stimulated cultures were maintained in a humidified 37°C, 5% CO2 atmosphere in an air incubator for seven days. Kinetic studies were first done to determine the optimal incubation time at which to assay for Ig secretion.

Immunoiglobulin assay. The amount of Ig in culture supernatants was measured using an enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (96-well) (Immulon 1, Dynatech, Alexandria, VA) were coated with 50 μL/well of affinity-purified goat anti-human Ig (Tago) at a concentration of 10 μg/mL in phosphate-buffered saline (PBS). After the cell cultures were washed with PBS containing 0.05% NP-40 (Sigma), 50 μL of culture supernatants, serially diluted in 1% bovine serum albumin (BSA) in PBS, was added to the wells for one hour at room temperature. The plates were washed again, and 50 μL of horseradish peroxidase (HRP) conjugated, affinity-purified, goat anti-human κ or λ light chain-specific antibodies (Tago) was added for one hour at room temperature. After the cultures were washed, 100 μL of a solution of 2, 2'-azino-di-(3-ethyl-benzthiazoline-sulfonate) (ABTS, Sigma) in 0.05 mol/L of sodium citrate buffer pH 4.0 with 0.01% hydrogen peroxide was added. Color development was determined by measuring the optical density (OD) at 405 nm using an MR600 microplate reader (Dynatech). From the mean OD, the amount of Ig was calculated from a standard curve produced using purified human IgGs. Although some assays were done on different days, the amount of Ig was always calculated based on simultaneously run standards. The sensitivity of this assay was 0.01 μg/mL.

Assay for IgM κ and λ light chains. Polystyrene microtiter plates were coated with 10 μg/mL of goat anti-human IgM (Tago) in PBS. The plates were washed as above and then incubated for one hour at room temperature with 5% instant milk (Carnation Nonfat Dry Milk, Los Angeles) in PBS. This blocking step was found to increase the sensitivity of the assay greatly by reducing the background. After another wash, the patient's sera were added by doubling dilutions with PBS containing 1% BSA (PBS–BSA) into 16 wells. The plates were incubated for one hour at room temperature and washed again. Horseradish peroxidase-conjugated affinity-purified goat anti-human κ or λ light chain-specific antibodies (Tago) were added. After one-hour incubation at room temperature and another wash, an ABTS solution was added and color development was measured as described above. This assay was performed simultaneously on the test samples and pooled normal human serum (HS, eight donors). Titration curves were generated, and the 50% saturation points were compared. The serum dilutions at these points were expressed as a percentage of the normal pooled HS. The amounts of IgM κ, relative to pooled normal HS, was then compared with the amount of IgM λ, relative to pooled normal HS.

Anti-idiotype antibodies. Murine monoclonal anti-idiotype antibodies were produced as previously described.37 Either purified idiotypic protein or whole tumor cells were used to immunize mice used in the making of anti-idiotype antibody-secreting hybridomas. The candidate anti-idiotypes were screened for nonreactivity with three different purified human IgM proteins and human tonsil. The antibodies used in these studies were purified from ascites.

Measurement of tumor-specific serum idiotype. An ELISA assay used to measure the concentration of serum idiotype was described previously.5 The microtiter plates (Dynatech) were coated with specific anti-idiotype antibody at a concentration of 10 μg/mL in PBS-NP-40. After the plates were washed, idiotype protein standard and unknown samples were serially diluted with PBS-BSA in parallel on the plate, incubated, and washed again. Biotin-conjugated specific monoclonal anti-idiotype antibody was added, incubated, and washed from the plate. Avidin conjugated to HRP (Vector, Burlingame, CA) was added at an appropriate dilution, incubated, and washed. ABTS was added as a final step, and the results were evaluated as in the immunoglobulin assay described above.

RESULTS

Characteristics of patients and tissues. Table 1 lists the 18 patients selected for these studies. All patients had advanced disease with easily palpable lymphadenopathy in multiple anatomic sites and/or large numbers of tumor cells in their peripheral blood. Lymphoma patients had stage III or IV disease; leukemia patients had WBC counts >25,000/μL, and most cells were malignant. Eleven of the 18 patients had received prior treatment with chemotherapy and/or radiotherapy. No patient had received therapy within 1 month of tissue sampling or serum collection. The sources of tissue were lymph node in 10 patients, peripheral blood in 5 patients, and spleen in 3 patients who underwent splenectomy for diagnosis (C.B.) or because of cytopenias or symptomatic organomegaly (J.C., C.G.).

The surface Ig type was established by immunofluores-
ence staining and FACS analysis of cell suspensions. In each case, except that of D.H., a predominant light chain type was found. Thus, these cases were believed to be monoclonal. These results were confirmed on frozen tissue samples using an immunoperoxidase technique. In D.H., the peripheral blood lymphocytes expressed either $\kappa$ or $\lambda$ light chain in approximately equal ratios.

The percentage of malignant cells and infiltrating normal T cells within the tissue are also shown in Table 1. These percentages were determined using immunofluorescence on the FACS gated on the lymphoid population of cells. The degree of tumor involvement was $>50\%$ in the specimens of 17 of 18 patients. One patient’s (P.E.) biopsy had only 21% tumor cells, with a strikingly large number of normal T cells (76%).

**Secretion of tumor idiotype in vitro.** Cell suspensions from tumor specimens were cultured with or without PMA and/or PHA. The optimal concentration of PMA (10 ng/mL) and time to measure secreted Ig in the culture supernatant (day 7) were determined in preliminary studies. Subsequently, cumulative Ig secretion was measured after a seven-day culture period (Table 2). Four patients (T.G., B.J., C.J., and C.P.) had undetectable amounts of Ig in control and in stimulated cultures. Five patients (R.B., K.C., N.D., C.G., and D.S.) had low levels of spontaneous or induced Ig secretion. The remaining nine patients had higher levels of secreted Ig. Three patients (R.P., L.R., and M.V.) had no detectable spontaneous Ig secretion, but significant quantities were secreted on stimulation with PMA, PHA, or both. Five patients (J.C., P.E., D.H., B.L., and F.S.) had high levels of spontaneous Ig secretion only minimally affected by stimulation.

In all but one case, only a single type of secreted light chain was detected. This always matched the light chain type found on the surface of the tumor cells. In D.H., both $\kappa$ and $\lambda$ light chains were found in culture supernatants. Immunoglobulin gene rearrangement studies showed that patient D.H. was biclonal as was reported previously. His tumor was comprised of a mixture of $\kappa$ and $\lambda$-bearing clones.

**Correlation of secretion in vitro with serum idiotype level.** Table 2 also shows the amount of tumor idiotype measured in the serum of these patients at the time of tissue sampling. The levels of serum idiotype correlated with the spontaneous or induced secretion of Ig in vitro (Fig 1). Patients with spontaneous or induced levels of secreted Ig $\geq 1.0 \mu g/mL$ had high serum idiotype levels (mean $\pm$ SD 265 $\mu g/mL$ $\pm$ 172) as compared with patients with secretion in vitro $\leq 0.5 \mu g/mL$ (mean $\pm$ SD 3.7 $\mu g/mL$ $\pm$ 9.9). Distribution of serum idiotype levels between these two groups did not overlap. The measurement of spontaneous or induced Ig secretion in vitro accurately identified patients with high serum idiotype levels. There was no correlation between the numbers of normal T cells within the tumor sample and the amount of spontaneous or induced Ig secretion in vitro (Tables 1 and 2). Further analysis of T cell subsets revealed no consistent pattern (data not shown).

**Analysis of serum IgM for $\kappa$ and $\lambda$ light chain.** Most B cell lymphomas express IgM on their cell surface and would secrete antibody with that heavy chain idiotype. The total amounts of IgM $\kappa$ and IgM $\lambda$ in the serum of most of the patients were below those found in pooled normal serum. This finding is a result of the hypogammaglobulinemia that frequently exists in patients with B cell lymphoma. An assay that measured the concentration of $\kappa$ and $\lambda$ light chains in the IgM component of serum was developed. The values were compared with those in pooled normal human serum; a calibration of this assay is shown in Fig 2. Various concentrations of either a purified IgM $\kappa$ or IgM $\lambda$ paraprotein were added to pooled human serum and compared with the unspiked serum. A minimum level of paraprotein of 200 $\mu g/mL$ was detectable in this assay. Higher levels caused an

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### Table 2. Secretion of Tumor Id In Vitro and In Vivo

<table>
<thead>
<tr>
<th>Patient</th>
<th>No Mitogen</th>
<th>PMA</th>
<th>PHA</th>
<th>Serum Ig Level (μg/mL)</th>
<th>Serum k-λ Imbalance</th>
</tr>
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<tbody>
<tr>
<td>C.B.</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>1.0</td>
<td>100</td>
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<tr>
<td>R.B.</td>
<td>0.15</td>
<td>0.25</td>
<td>0.15</td>
<td>0.4</td>
<td>0.01</td>
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<tr>
<td>J.C.</td>
<td>1.4</td>
<td>0.7</td>
<td>1.4</td>
<td>1.3</td>
<td>&gt;150</td>
</tr>
<tr>
<td>K.C.</td>
<td>0.10</td>
<td>0.05</td>
<td>0.10</td>
<td>0.15</td>
<td>30</td>
</tr>
<tr>
<td>N.D.</td>
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<td>0.4</td>
<td>0.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>P.E.</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>150</td>
</tr>
<tr>
<td>T.G.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>No</td>
</tr>
<tr>
<td>C.G.</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>D.H.*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(κ)</td>
<td>0.6</td>
<td>1.8</td>
<td>0.6</td>
<td>1.0</td>
<td>NM</td>
</tr>
<tr>
<td>(λ)</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.6</td>
<td>NM</td>
</tr>
<tr>
<td>B.J.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
<td>No</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>No</td>
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<td>B.L.</td>
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<td>1.0</td>
<td>1.5</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>R.P.</td>
<td>0</td>
<td>0.6</td>
<td>1.5</td>
<td>3.0</td>
<td>&lt;500</td>
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<tr>
<td>L.R.</td>
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<td>1.2</td>
<td>1.2</td>
<td>&gt;200</td>
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<tr>
<td>D.S.</td>
<td>0</td>
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<td>0</td>
<td>0.05</td>
<td>&lt;1</td>
</tr>
<tr>
<td>F.S.</td>
<td>0.9</td>
<td>0.6</td>
<td>1.1</td>
<td>1.4</td>
<td>500</td>
</tr>
<tr>
<td>M.V.</td>
<td>0</td>
<td>1.0</td>
<td>0.6</td>
<td>1.1</td>
<td>400</td>
</tr>
</tbody>
</table>

Id, idiotype; PMA, phorbol myristate acetate; PHA, phytohemagglutinin. 0, none detectable; NM, not measured; NA, not applicable because tumor secreted IgG.

*Secretion of both light chain classes was observed. Values for both are listed.
increasingly greater excess of light chain in the test serum as compared with the unspiked serum. As expected, the nonparaprotein-associated light chain levels remained constant in all the test samples. Patients with IgG-secreting tumors were tested using a similar assay. The large amounts of paraprotein-associated light chain levels remained constant in all the test samples. Patients with IgG-secreting tumors were tested using a similar assay. The large amounts of normal IgG in serum obscured the results.

This assay was performed on serum from 14 patients, as shown in Fig 3. Seven patients (C.B., P.E., B.L., R.P., L.R., F.S., and M.V) had an imbalance in the amounts of light chains as compared with patients having normal serum. This is best demonstrated in patient R.P., who had a monoclonal IgM k protein spike detected by serum protein electrophoresis. Using this assay, we found more IgM k in the patient's serum than in the normal serum; however, the IgM λ level was quite low. This caused a dramatic imbalance in the levels of IgM light chains as compared with those in normal serum. Similar, but less dramatic, results were obtained in six other patients. Each of these seven patients had high levels (>100 μg/mL) of idiotype in the serum. The other seven patients had no significant imbalance in IgM light chains and had serum idiotype levels <15 μg/mL. As compared with the data shown in Fig 2, detectable levels of circulating monoclonal proteins were lower in the patient’s serum (100 μg/mL in C.B.) than in normal serum. This is likely related to the lower background IgM levels, which made it easier to detect small amounts of the monoclonal Ig. This assay successfully predicted which patients secreted ≥100 μg/mL of idiotype into the serum, provided that the idiotype was IgM.

DISCUSSION

Because a custom-made antibody must be made for each patient, criteria must be established that will facilitate the selection of the most favorable candidates for anti-idiotype therapy. Circulating antigen interferes with therapy using antibodies.4,6,9 This has been a particularly important problem in studies using anti-idiotype antibodies directed against the Ig present on B cell tumors.4,6,9 Patients with insurmountable levels of idiotype in their serum should be excluded from therapeutic trials. In the past, an anti-idiotype antibody first had to be produced; it could then be used to measure the level of circulating idiotype protein. In this study, we developed in vitro assays that predict which patients have significant levels of serum idiotype without using an anti-idiotype reagent.

We prepared monoclonal anti-idiotype antibodies for all the patients studied in this report except D.H. This enabled us to measure idiotype levels accurately in these patients to establish a correlation with in vitro predictive tests. Several factors may have influenced both the in vitro assay results and the in vivo idiotype levels. The number of cells added to each well of the in vitro assays was based only on viable cells, with no adjustments made for the cellular composition, ie, the ratio of tumor, normal B cells, and normal T cells. Thus, the number of Ig-secreting cells in each well were proportionately reduced in tumors with large numbers of T cells. In addition, the T cells may influence, either positively or negatively, the ability of the malignant B cells to secrete Ig. Finally, the in vivo level of serum idiotype varies directly with tumor bulk.11 Despite these theoretical problems, the in vitro assays reliably predicted in vivo levels.

The effects of various mitogens, including PHA and phorbol esters, on secretion of immunoglobulin by normal and neoplastic B cells were previously studied in vitro.20-28 PHA added to cultures of normal T and B cells caused proliferation of both sets of cells, expression of interleukin 2 (IL2) receptor on the T cells, and increased secretion of Ig by B cells. Most of these effects were mediated by soluble B cell growth and differentiation factors secreted by the stimulated T cells. The mitogens produced both increased secretion of Ig and maturation of the malignant cells, as measured by switch of idiotype-positive IgM and IgD to IgG.21,22 No correlation with the in vivo secretion of the malignant cells has been previously reported. Although a direct effect of the mitogens on the tumor cells produced some enhancement of secretion, their effect on T lymphocytes, also present in the culture, was probably more important.23 Defects in the in vivo function of the T lymphocytes of patients with B cell malignancies have been identified.23-25 Some reports showed that allogeneic cells were far better at stimulating secretion of Ig than were autologous T cells.22,23,26 Indeed, suppression was induced by adding back autologous T cells to a culture containing normal allogeneic T cells and the malignant B cells.26 Other
investigators were unable to identify abnormal T cell suppression.27,28 We found no correlation between the numbers or types of T cells and spontaneous or induced Ig secretion among the tumors in this study.

The autologous T cells may have suppressed production in the patients whose tumors secreted little or no Ig in vitro or in vivo. Among those that secreted Ig in vitro, however, our data suggested an enhancing effect of T cell stimulation on Ig secretion. Differences were evident among the patients regarding the necessity for PHA and/or PMA to produce maximal secretion of Ig. In patients R.P. and L.R., PMA and PHA had a synergistic effect on idiotype secretion. PMA induces IL 2 receptor expression on T cells.29 This effect, together with the T cell proliferation induced by PHA, may have led to amplification of synthesis and secretion of B cell growth and differentiation factors. Patient R.P. had the highest level of secretion in vitro (Table 2, >3.0 µg/mL), but only after stimulation with both PMA and PHA. Serum protein electrophoresis (SPEP) showed that this patient had a paraprotein spike of 20 mg/mL, suggesting that T cells were necessary for tumor idiotype secretion in vivo as well as in vitro. Similar observations were made for L.R. and M.V., who also had high levels of serum idiotype. Previously, we reported that the absolute number of T cells in the tumor correlated positively with the patient’s response to anti-idiotype therapy.15 T cells located within the tumor or elsewhere may regulate the in vivo secretion of idiotype as well as the control of growth and differentiation of the malignant B cells.

The assay to measure spontaneous and induced secretion of Ig was designed to correlate with in vivo secretion and thus with serum idiotype. The effects of autologous T cells were not eliminated in this assay, so that we could simulate the in vivo situation. Although this assay measured free light chains and IgG as well as IgM, we believe that quantitation of the total Ig secreted in vitro is important in determining the in vivo secretory capacity of the tumor cells. The assay was successful in this respect. The spontaneous or induced secretion of idiotype was able to identify patients with high serum idiotype levels (Fig 1). In vitro secretion <0.5 µg/mL can be used to select patients with little or no circulating idiotype. Conversely, in vitro secretion of >1.0 µg/mL identifies patients with high levels of serum idiotype who are not good candidates for anti-idiotype therapy.

We also tested an assay that measures the relative imbalance of IgM light chains in the serum to detect circulating IgM tumor idiotype. The advantage of this test is that culture of cells is not required and results may be obtained rapidly. Although the assay successfully identified patients with large amounts of IgM, its lack of sensitivity and inapplicability to IgG-secreting tumors limited its usefulness.

Other workers have isolated tumor idiotype either from serum or from cultures of malignant cells.30 Although these techniques simplify the process of making anti-idiotype antibodies, it requires patients with high levels of serum idiotype. Thus, these methods have the disadvantage of selecting for the most unfavorable patients.

Our studies have considerable practical significance. The ability to select patients with low levels of serum idiotype will facilitate therapeutic studies using anti-idiotype. Our studies may also be useful in monitoring tumor activity and response to treatment. In addition to their practical importance, our studies have important implications regarding the immunoregulation of B cell tumors.

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In vitro tests that predict tumor-associated idiotype levels in the serum of patients with B cell lymphomas and leukemias

RA Miller, JN Lowder, J Gralow, T Meeker and R Levy