Lymphoma Regression Induced by Monoclonal Anti-Idiotypic Antibodies Correlates With Their Ability to Induce Ig Signal Transduction and Is Not Prevented by Tumor Expression of High Levels of Bcl-2 Protein

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Custom-made monoclonal anti-idiotypic antibodies (anti-Id MoAbs) have been tested as a treatment modality in 34 non-Hodgkin's lymphoma (NHL) patients. Partial or complete tumor remissions have been induced with this treatment in 68% of these patients. One mechanism by which anti-idiotypic antibodies may have induced these tumor responses is via a direct antiproliferative effect on the tumor cells, resulting in apoptosis. Primary NHL cells do not proliferate well enough in vitro to test this hypothesis directly. Therefore, we studied the effect of anti-idiotypic antibodies on signal transduction through the surface Ig receptor as measured by the induction of cellular protein tyrosine phosphorylation. To assess whether bcl-2 protein could protect lymphoma cells from death induced by anti-Id MoAb, we also measured the level of bcl-2 protein in the same tumor cells. We found a strong correlation between the ability of an anti-Id MoAb to induce an increase in tyrosine phosphorylation in vitro and its ability to induce a tumor regression in the patient. By contrast, the level of bcl-2 expressed by the tumor cells was not correlated with clinical response to anti-Id MoAb treatment.

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The majority of non-Hodgkin's lymphomas (NHLs) are characterized by a monoclonal expansion of surface Ig expressing B lymphocytes. The idiotype of the Ig on these tumors is a tumor-specific marker and has been used as an immunotherapeutic target. Treatment with anti-idiotypic murine monoclonal antibodies (anti-Id MoAbs), either alone or in combination with other treatment modalities such as interferon-α (IFN-α) or chlorambucil, have induced partial or complete remissions in 68% of these patients.1 The mechanism of this anti-Id MoAb-induced antitumor effect remains unknown. It is generally accepted that host effector systems (such as complement or antibody-dependent cellular cytotoxicity [ADCC]) that interact with the Fc portion of the antibody are required for the generation of an antitumor effect. Studies of immunotherapy in murine systems, using human or murine tumor cells, have shown that the isotype of the Ig heavy chain of the murine MoAb determines whether antibody-coated tumor cells will be killed.5,8 In our clinical trials, the majority of the murine anti-Id MoAbs were of the IgG1 isotype, which does not interact with human complement to lyse cells or mediate ADCC. Furthermore, there was no correlation between the isotype of the MoAb used for therapy and the clinical response.9

Alternatively, antibodies or antigens that bind to the cell surface Ig receptor can mediate a direct inhibition of cell growth.10-12 This negative growth signal has been shown to be dependent on the tyrosine phosphorylation of a set of critical intracellular proteins.13 With rare exceptions,8 it has not been possible to test our anti-Id MoAbs for antiproliferative effects directly because of the inability to induce primary tumor cells from lymphoma patients to grow in vitro. However, with the development of murine MoAbs that bind phosphorylated tyrosine residues,14 it has become possible to study protein tyrosine phosphorylation as a measure of signaling through the Ig receptor. We studied the direct effects of the anti-Id MoAbs on tumor cells that had been cryopreserved from the pretreatment biopsy specimens of patients treated in our anti-Id MoAb clinical trials. We found that the respective anti-Id MoAbs increased protein tyrosine phosphorylation only in tumors from patients that had clinically responded with a partial or complete remission to the anti-Id immunotherapy.

Certain intrinsic properties of the tumor cell might determine its response to growth-inhibiting signals such as anti-Id antibodies. One of these is the level of the bcl-2 protein. This 24-kD protein,15-17 which is expressed during critical stages of normal B-cell differentiation,18,19 is deregulated by the t(14; 18) translocation that occurs in human follicular lymphoma.20,21 Although the biochemical function of the bcl-2 protein is still unknown, there is increasing evidence that it can protect normal B lymphocytes from programmed cell death, apoptosis.22-24 Its presence has recently been shown to protect cell lines even from apoptosis induced by nitrogen mustard and corticosteroids.25 Most of the NHL patients in our antibody trials had follicular lymphoma containing the t(14;18) chromosomal translocation. Therefore, we measured the levels of bcl-2 protein in these patients' tumor cells to determine whether responding and nonresponding patients could be distinguished by this parameter. The results showed that, although the expression of bcl-2 protein was quite variable among the tumors from different patients, the level of expression of this protein did not correlate with tumor responses induced by anti-Id antibodies.

Materials and Methods

Tumor cells and anti-Id MoAb. The tumor cells in this study were from patients in our three published clinical trials with anti-Id MoAb used alone or in combination with IFN-α or with chloram-

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bucil.1 Anti-Id MoAb reactive with private idiotypes expressed by the tumor cells of these patients were produced, purified, and used for therapy. Before immunotherapy, suspensions of tumor cells had been isolated from lymph node or spleen specimens and cryopreserved, affording us the unique opportunity to study pretreatment tumor cells from patients whose response to therapy was known. For analysis, the tumor cells were thawed, washed twice with culture medium (RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin), and then incubated for 1 to 2 hours at 37°C. Although some of the tumor cells had been cryopreserved in liquid nitrogen for more than 10 years, the viability as determined by trypan blue dye exclusion at the time of analysis exceeded 80% in all cases.

**Phosphotyrosine detection by immunoblotting.** Tumor cells (2 × 10⁶) were incubated in vitro at 37°C in a volume of 1 mL with medium, anti-Id MoAb, or anti-lg for 10 seconds to 15 minutes. Negative control treatments were performed with a nonbinding anti-Id MoAb or with a binding but nonsignaling anti-HLA class I MoAb (W6/32; Serotec, Kidlington, UK). A positive control was performed using goat polyclonal antibodies to human IgM (Jackson Immunoresearch Laboratories, West Grove, PA) or goat polyclonal antibodies to human IgG (Tago, Inc, Burlingame, CA). After the antibody treatment, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing 1 mmol/L Na₂HPO₄. Cell pellets were lysed for 1 to 2 hours at 4°C in 100 μL lysis buffer containing 1% Triton-X100, 150 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L Na₂HPO₄, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, and 1 μg/mL aprotinin. Cell lysates were centrifuged for 15 minutes at 15,000 rpm, and 20 μL of the supernatant was mixed with 10 μL 3X sample buffer (187.5 mmol/L Tris, 9% sodium dodecyl sulfate (SDS), 30% glycerol, and 0.4 mg/mL bromphenol blue) and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using acrylamide concentrations of 8% or 10%.

**RESULTS**

**Bcl-2 expression in primary NHL cells.** We determined the expression of bcl-2 in the pretreatment tumor cells from patients who had been treated with anti-Id MoAb. Bcl-2 expression was determined by flow cytometry, using fixed and permeabilized cells to allow staining of the intracellular protein. Because of the ubiquitous expression of bcl-2 in both the malignant and normal cells in the biopsy specimens, tumor cells were identified by costaining with anti-light chain Ig reagents labeled with a second fluorochrome.

An example of this two-color flow cytometric analysis is shown in Fig 1. Using this approach, the PE signal (mean channel PE fluorescence) was detected using a murine IgG1 antibody (Phar ma, Carpintena, CA). After washing, the bound murine IgG1 antibody was detected with PE-goat antimurine IgG (Tago). Stained cells were fixed in PBS containing PBS and 0.05% sodium azide. Aliquots of 1 × 10⁶ cells were then stained for flow cytometry. Intracellular bcl-2 was detected using a murine IgG1 antimurine bcl-2 MoAb (0.025 mL of a 1/100 dilution, Dako BCL-2; 124, Dako Co, Carpintena, CA). After washing, the bound murine IgG1 antibody was detected with PE-goat antimurine IgG (Tago) and fluorescein isothiocyanate (FITC)-labeled anti-CD20 (Leu 16) and anti-CD8 (Leu 2) (Beckton Dickinson, San Jose, CA). For indirect staining, the bound first-step murine MoAb was detected with FITC-labeled F(ab’); goat antimouse Ig (Tago). Stained cells were fixed in PBS containing 2% paraformaldehyde, were stored at 4°C, and were analyzed for two-color staining with a FACS 440 (Beckton Dickinson).

**Intracellular staining for bcl-2 protein.** Cryopreserved tumor cells from lymph-node or spleen tissues or peripheral blood lymphocytes were washed and centrifuged over a Ficoll-Paque (Pharmacia, Piscatway, NJ) gradient. Viable cells were collected and washed twice in PBS. Cells were fixed and permeabilized according to slight modifications of a previously published method. Briefly, 5 × 10⁶ tumor cells were resuspended into 1 mL of 2% paraformaldehyde in PBS and incubated on ice for 10 minutes. Triton X100 (0.1 mL of a 0.5% solution) was added, and the mixture was incubated for an additional 10 minutes on ice. The cells were then washed once with PBS and once with PBS containing 1% BSA and 0.05% sodium azide. A quench of 1 x 10⁶ cells was then stained for flow cytometry. Intracellular bcl-2 was detected using a murine IgG1 antimurine bcl-2 MoAb (0.025 mL of a 1/100 dilution, Dako BCL-2; 124, Dako Co, Carpintena, CA). After washing, the bound murine IgG1 antibody was detected with PE-goat antimurine IgG (Tago) and fluorescein isothiocyanate (FITC) labeled anti-CD20 (Leu 16) and anti-CD8 (Leu 2) (Beckton Dickinson, San Jose, CA). For indirect staining, the bound first-step murine MoAb was detected with FITC-labeled F(ab’) goat antimouse Ig (Tago). Stained cells were fixed in PBS containing 2% paraformaldehyde, were stored at 4°C, and were analyzed for two-color immunofluorescence. The mean channel bcl-2 fluorescence of the tumor cells was determined by analyzing the PE signal from the cells expressing the correct light chain.
levels of bcl-2 expressed by the patients' tumor cells and their clinical outcome in response to anti-Id MoAb therapy are shown in Fig 2. There was no difference between the level of bcl-2 expression in patients who did or who did not respond to the antibody therapy. Significant antitumor effects were observed in some patients whose tumor cells expressed relatively high levels of bcl-2 protein, as well as in some patients whose tumor cells expressed low levels of bcl-2.

Detection sensitivity of anti-Id MoAb-induced increase of protein tyrosine phosphorylation. The cell suspensions from biopsy specimens contained variable percentages of tumor cells and normal mononuclear cells. In preliminary experiments, it was necessary to determine whether the presence of normal cells would interfere with the ability to detect the induction of tyrosine-phosphorylated proteins in the tumor cells. Cells from a splenic tumor sample from 1 patient (containing 50% idiotype-positive B cells) were mixed with normal peripheral blood mononuclear cells in various ratios, resulting in mixtures containing from 100% to 25% spleen cells. Aliquots of each mixture containing 2 X 10^6 cells were incubated with various antibodies for 2 minutes at 37°C and then analyzed for the degree of tyrosine phosphorylation that resulted from that treatment. The result of a representative experiment is shown in Fig 3. The cell mixtures were treated with antibodies, as indicated at the top of Fig 3A, for 2 minutes at 37°C. Baseline tyrosine phosphorylation was determined using an irrelevant anti-Id MoAb that did not bind to the cells and an anti-HLA class I MoAb (W6/32) that did bind to the cells but would not be expected to trigger them. Lysates were run on 10% SDS-PAGE and blotted to nitrocellulose. The blot was first stained with "second step only" (Fig 3B), and phosphorylated tyrosine residues were subsequently detected with the antiphosphotyrosine-specific MoAb 4G10. Figure 3A shows that the increase in tyrosine-phosphorylated proteins could be detected even when the tumor biopsy specimen contained as few as 25% spleen cells (representing 12.5% idiotype-positive tumor cells). Staining with "second step only" (Fig 3B) shows that the amount of a background protein of 80 kD, which presumably binds streptavidin/HRP, was comparable between the different lanes, confirming that equal amounts of protein were loaded in each lane of the gel. Furthermore, Fig 3B shows that by decreasing the tumor cell content of the activated cell suspension, the amount of murine anti-Id MoAb that was carried over in the lysate (position of the mouse MoAb heavy chain is indicated with an
The cells from patient RB showed an increase of protein tyrosine phosphorylation as soon as 10 seconds after exposure to the relevant anti-Id MoAb. However, the tumor cells from patient ID showed no increase in tyrosine phosphorylation over background (medium-treated cells) after exposure to the anti-Id MoAb. The film shown in Fig 4A was analyzed with a densitometer, and the resulting histogram of this analysis is shown in Fig 4B.

A summary of the results obtained with the tumor cells from all 26 patients is shown in Fig 5. Of the 8 patients from the nonresponder group, none showed significantly increased tyrosine phosphorylation after exposure to tumor-specific anti-Id MoAb, whereas the tumor cells from all of these nonresponding patients showed some increased tyrosine phosphorylation in response to polyclonal anti-Ig activation. An increase of tyrosine phosphorylation after exposure to the relevant anti-Id MoAb was observed in the tumor cells from the patients that showed a partial regression or a complete regression of their tumor after anti-Id MoAb treatment. Polyclonal anti-Ig showed a greater stimulation in these clinically sensitive tumors than in the clinically insensitive tumors. It is also clear from these results that the increase in tyrosine phosphorylation was not different between the partial and complete responder groups.

**DISCUSSION**

In this report, we show a correlation between the ability of anti-Id MoAb to increase tyrosine phosphorylation in vitro and to induce a clinically significant tumor regression in
the patient. By contrast, the levels of bcl-2 expressed by the tumor cells did not correlate with treatment outcome.

We studied a total of 24 patients for the levels of bcl-2 expression by pretreatment tumor cells. From the results (Figs 1 and 2), it is clear that the level of bcl-2 expression between different tumors was quite variable, but that there was no correlation with anti-Id MoAb treatment outcome. Our data are in contrast to published studies that show that bcl-2 protects cells from chemotherapeutic drugs, growth-factor deprivation, or glucocorticoid-induced cell death.\textsuperscript{25,28,29} However, they appear to be consistent with recent observations regarding the lack of the ability of transfected bcl-2 to protect from anti-IgM–induced cell killing in the WEHI-231 cell line.\textsuperscript{30} In this system, bcl-2 prevented apoptosis induced by heat shock but not apoptosis induced by anti-Ig signaling. Thus, although the presence of high levels of bcl-2 in patients with follicular lymphoma and the t(14;18) translocation could possibly protect the cells from a variety of insults including chemotherapy, they may remain vulnerable to therapies targeting the surface Ig.

The immunoblot assay used to detect phosphorylated tyrosine residues was able to detect maximal increases in tyrosine phosphorylation induced by anti-Id MoAb of a cell suspension that contained as few as 12.5% Ig-positive cells (Fig 3). This may be caused by the fact that the tyrosine-phosphorylated proteins generated in the mixture containing 12.5% Ig-positive tumor cells exceeded the amount necessary to saturate the assay. An alternative explanation would be that activated protein tyrosine kinases from the tumor cells, after solubilization in lysis buffer, phosphorylated tyrosine residues of proteins that originate from the normal mononuclear cells. In either case, the signal measured is a consequence of specific interaction with the tumor cell surface Ig receptor. The induction of protein tyrosine phosphorylation with anti-Id MoAb in primary lymphoma cells followed similar kinetics to that previously published for polyclonal anti-IgM antibodies on murine and human B-cell lines.\textsuperscript{31-34} Furthermore, our data also showed that there was no difference in the increase of tyrosine phosphorylation between patients who responded with a partial or complete tumor remission after anti-Id treatment. This suggests that tyrosine phosphorylation may be necessary for the induction of an antitumor response, but that other subsequent events in the host determine the degree to which the tumor will regress.
Fig 5. Correlation between antibody-induced tyrosine phosphorylation and clinical response in 26 anti-Id MoAb-treated patients is shown. After immunoblotting for protein tyrosine phosphorylation, the x-ray films were analyzed by densitometer. The signal in anti-Id-treated and anti-lg lanes compared with the control lanes was expressed as a ratio (SI). The clinical response of the patient to anti-Id MoAb therapy is indicated in the top left of each graph: no or minor response, NR/MR; partial remission, PR; and complete remission, CR.

The perfect correlation in this study between in vitro signal transduction and clinical effects of anti-Id antibodies is surprising because a variety of other host factors are known to interfere with the clinical effects. For instance, in some patients, we have shown the emergence of Id-negative variant lymphoma cells after treatment with anti-Id MoAb. The analysis of a lymph-node sample, taken after clinical relapse after anti-Id treatment and containing only Id-negative cells, confirmed that exposure of these cells to the treatment anti-Id did not stimulate protein tyrosine phosphorylation (data not shown), whereas the Id-positive tumor cells from a pretreatment biopsy specimen did show an increased tyrosine phosphorylation. In addition, the development of a human antimouse Ig response or high levels of soluble Id protein present in the serum has been shown to limit the ability of anti-Id MoAb to reach the tumor cells. In such patients, anti-Id-MoAb exposure of tumor cells might show an increase in protein tyrosine phosphorylation in vitro, but MoAb treatment would not be expected to induce a tumor regression in vivo.

The induction of tyrosine phosphorylation by cross-linking of surface Ig on B lymphocytes has been shown to be an initial step in a cascade of events leading to programmed cell death or apoptosis, resulting in DNA degradation. Our own experiments performed with a clonotypic MoAb against the T-cell receptor expressed by a leukemic T-cell line showed that the MoAb did induce cell death by DNA fragmentation.

Whether the anti-Id MoAb-induced protein tyrosine phosphorylation will prove to be more characteristic of the particular anti-Id MoAb or of the tumor target cells is not known. In 1 patient, three of four different anti-Id MoAbs, all of which bound to a similar percentage of tumor cells, were able to induce a similar increase in tyrosine phosphorylation (data not shown). This would indicate that in this 1 case the antibodies were not identical in their ability to induce signal transduction. However, the tumor cells from patients may differ in their intrinsic ability to transduce the anti-Id MoAb signal, as shown by the finding that increased tyrosine phosphorylation induced by polyclonal anti-lg antibodies was generally less efficient in nonresponder-patient tumor cells compared with tumor cells from patients who responded to the antibody therapy (Fig 5). Furthermore, an MoAb against human IgM (1D12) seemed to increase tyrosine phosphorylation in preliminary experiments only in responder tumor cells. These results suggest that the intrinsic ability of the tumor cell to be triggered through the Ig receptor may also play a role in clinical outcome to anti-Id MoAb therapy. The question that remains is whether an anti-Id MoAb could be made which would induce signal transduction in the cells of the nonresponder patients. The availability of anti-Id MoAb against shared idiotypic determinants that do not have to be custom-made for each patient may provide a means to elucidate the above question. It will be possible to study the induction of tyrosine phosphorylation by these anti-Id MoAbs on the tumor cells from more than 1 patient.

Taken together, our results suggest that in the future anti-Id MoAbs which are selected for treatment should not only bind to the lymphoma target but also be able to induce an increase in cellular protein tyrosine phosphorylation after binding their target antigen.

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Lymphoma regression induced by monoclonal anti-idiotypic antibodies correlates with their ability to induce Ig signal transduction and is not prevented by tumor expression of high levels of bcl-2 protein

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