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Studies on B Lymphoid Tumors Treated With Monoclonal Anti-idiotype Antibodies: Correlation With Clinical Responses

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Monoclonal anti-idiotype antibodies can be made which are exquisitely specific for B lymphocytic malignancies. We have conducted a clinical trial in which some patients' tumors regressed after infusion of such antibodies. Here, we evaluated characteristics of the antibodies, the tumors, and the patients to determine which features best correlated with the clinical response. Neither the isotype of the murine antibodies, nor their avidity were predictive of clinical outcome. The specific epitope to which the antibodies bound was characterized by immunochemical techniques. Reactivity with a heavy-light chain combinatorial determinant correlated somewhat with clinical effect. Variations in the characteristics of the individual tumors such as antigen sites per cell and ability to modulate the surface immunoglobulin were not predictive of response. In one patient with prolymphocytic leukemia the anti-idiotype antibody had a direct antiproliferative effect on tumor cells in vitro. This patient's tumor response was explainable by such a direct mechanism. In the other patients, who had lymphomas, therapeutic outcome correlated with the number of host nontumor cells infiltrating the tumor. The vast majority of these nontumor cells were mature T lymphocytes of the Leu 4, Leu 3 (T3, T4) phenotype. Thus, a preexistent host-tumor interaction seems to be important in the in vivo effect of anti-idiotype antibodies in B cell tumors.

THERAPY WITH MURINE monoclonal antibodies (MoAbs) directed against human tumor cell surface antigens has been tested in several clinical trials.[1-12] Antibodies specific for the idiotype of the surface immunoglobulin (Ig) of B cell malignancies have been among the most successful in inducing regressions of tumor.[1,3] In our clinical trial, six of ten evaluable patients experienced clinically significant responses of disease during MoAb therapy. One patient's disease regressed completely. Of the five others who experienced a partial response, four had virtually all the tumor which reacted with the antibody eradicated. In those four cases, the original tumor population was replaced by mutant tumor cells which were unreactive with the antibody.[13,14] However, four other patients treated with anti-idiotype antibodies had no tumor response. It is important to determine which variables pertaining to the antibodies, tumors, or patients may have influenced the clinical outcome. We previously described a correlation between the numbers of normal T cells infiltrating the tumor with response to anti-idiotype antibody therapy.[15] Other variables may also be important. A unique antibody was developed for each patient. Thus, the antibodies potentially differed from each other in isotype, in avidity of binding, and in epitope specificity.

In addition to the properties of the antibodies, differences in the tumors are potentially important. Normal B lymphocytes are responsive to many regulatory substances which promote or inhibit proliferation and/or differentiation.[16-23] Anti-Ig or specific antigen, which, like anti-idiotype antibodies, bind to the surface immunoglobulin, can stimulate or inhibit the proliferation of normal B cells, even in the absence of other mitogenic factors.[16-18,23,24] Jerne proposed that autologous anti-idiotype antibodies exert a major inhibitory force on B cell clones in the immune response.[25] In addition, the maturational state of B cells greatly influences their dependence on growth factors and their receptiveness to immune control, including that mediated by anti-Ig antibodies.[16,17] B cell tumors appear to represent various stages of normal differentiation, reflected by their morphologic appearance, ability to secrete immunoglobulin, and tissue localization. By analogy, the differentiation state of a tumor may alter its response to anti-idiotype antibody.

Patients with B cell lymphoma can be immune deficient as a consequence of their disease and/or treatment.[3,26,27] Anergy, frequent infections, hypogammaglobulinemia, and cytopenias are common problems in patients with B cell malignancies, but vary in severity from patient to patient. These patients might thus have effector systems for antibody-mediated tumor responses. The natural history of low grade B cell lymphoma is characterized by long periods of quiescent disease and occasionally spontaneous regressions.[28] Some host antitumor response may be operative in these patients and the anti-idiotype antibody might augment that interaction.

Thus, analysis of the factors important in determining the outcome of anti-idiotype treatment is complicated by the large number of variables. Each patient, his tumor, and his antibody are unique. In this study, we attempted to correlate a variety of in vitro studies of the antibody, tumor, and patient with the clinical response.

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MATERIALS AND METHODS

Patients

Patients in this study and their treatment with anti-idiotype antibody were described in detail in a prior publication.[3] Briefly, all patients had B lymphoid malignancies, eight with nodular lymphoma and one each with large cell lymphoma, prolymphocytic leukemia, and diffuse small cleaved cell lymphoma with foci of large cell lymphoma. Monoclonal anti-idiotype antibodies were made for each patient's tumor as previously described.[29] Tumor samples obtained from the peripheral blood, bone marrow, lymph nodes, skin masses, or spleen of each patient had been frozen in liquid nitrogen and were available for this study either as viable cell suspensions or as frozen tissue fragments.

Immunophenotyping and Enumeration of Subsets

Tumor samples taken prior to therapy were evaluated by routine pathologic analysis, immunoperoxidase staining of frozen sections, and immunofluorescence analysis of single cell suspensions using methods that have been previously described.[30-33]

Rescue Hybridization

In order to obtain idiotypic protein from the nonsecreting tumor cells, "rescue fusions" were performed with mouse myeloma or heteromyeloma cells as previously described.[34,35] The resulting hybrids were screened for secretion of human heavy and light chains of the appropriate type by enzyme-linked immunosorbent assay (ELISA). The secreted proteins were purified by immunoaffinity chromatography and used in the assays described below.

Quantitation of Surface IgM

The relative amount per cell of surface immunoglobulin on the different tumors was obtained by flow cytometry using the FACS 440 fluorescence-activated cell sorter (Becton Dickinson, Mountain View, Calif) by methods previously described.[3] The mean linear fluorescence intensity[36] was determined on single cell suspensions of tumor stained with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody, 1D12, which is reactive with human μ heavy chain.

Binding constant determination

Equilibrium plate method. Anti-idiotype antibody was metabolically labelled with ^{75}Se-selenomethionine (Amersham Co, Arlington Heights, Ill). Approximately eight million hybridoma cells were incubated for 16 hours in methionine-free RPMI 1640 (GIBCO, Grand Island, NY) containing 5% dialyzed heat-inactivated fetal calf serum. After the labelling period, cells were removed by centrifugation and the supernatant was dialyzed extensively against phosphate buffered 0.9% saline (PBS) to remove unincorporated label. The concentration of anti-idiotype antibody in the labelled supernatant was determined by immunoassay. The percent of protein-bound radioactivity in the form of active antibody was determined by binding at equilibrium to a vast excess of idiotype (tumor cells) or goat anti-mouse Ig (coupled to Sepharose beads). The fraction of the total radioactivity that was bindable varied from 24% to 50%.

The binding constant determination was performed in a microtiter plate (Cooke Engineering, Alexandria, VA). Wells were coated with 100 μL of diluted idiotype protein or an irrelevant IgM and then incubated with 150 μL of PBS/bovine serum albumin (BSA) for one hour. After washing, 100 μL of serially diluted labelled anti-idiotype was added to the wells. After a six-hour incubation at room temperature, the supernatants were removed and the plates were quickly washed four times. The dry wells were cut with a hot wire device and counted in a gamma counter. All experimental points were measured in triplicate.

The free counts in each well were determined by subtracting the bound counts from the total input counts. The ratio of bound/free was plotted v bound and a slope of their curve was determined,[37] and the binding constant was determined according to the following equation:

$$K = \frac{-(\text{slope})(SA)10}{4.44(\text{mol wt})} \times 1.4(\text{vol}) \qquad \begin{array}{l} SA = \text{cpm}/\mu g \\ \text{vol} = 0.1\ \text{mL} \\ \text{mol wt} = 150{,}000 \end{array}$$

Soluble complex method. The second method used to measure antibody avidity was a modification of the method reported by Griswold and Nelson.[38] This method is based upon thermodynamic principles which predict that the binding of antibody to antigen will decrease as the total reaction volume is increased, provided the number of moles of antigen and antibody are held constant. The rate of this decrease is related to antibody avidity.

The assay was performed by mixing anti-idiotype with a 3-fold excess of idiotype, and diluting this mixture 1:5 and 1:10 into PBS/10% fetal calf serum. After a four-hour incubation, 50 μL aliquots of these mixtures were added, in quadruplicate, to wells of a 96-well microtiter plate coated with goat anti-mouse IgG antibody. The concentration of mouse anti-idiotype initially mixed with idiotype was previously determined to be nonsaturating on the anti-mouse IgG coated plate. After another four-hour incubation, plates were washed and a horseradish peroxidase (HRP)-coupled goat anti-human IgM antibody was added followed by 2,2'-azinodi-(3-ethylbenzthiazoline) sulfonic acid (ABTS) substrate to detect anti-idiotype*idiotype complexes bound to the plate. Avidity was calculated utilizing the BASIC program described by Griswold and Nelson.[38] Given the initial idiotype concentration, the dilution factor, and the fraction idiotype bound at two different reaction volumes the program determines an avidity value using a binary search algorithm.

Western Blots

Probing of purified proteins with radioactive antibodies was performed following the method of Burnette.[39] Idiotype protein from each patient was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system in both reduced and unreduced states. The protein was electrophoretically transferred to nitrocellulose (0.45 μm, Millipore, Bedford, MA) overnight. The nitrocellulose was incubated 2 to 8 hours in PBS/5% nonfat dry milk (Carnation, Los Angeles) (PBS/NFDM). First stage antibody at 5 to 10 μg/mL in PBS/NFDM, was followed by an ^{125}Iodine-labelled goat anti-mouse Ig (TAGO, Burlingame, Calif) also in PBS/NFDM. The nitrocellulose was washed between steps with 0.9% saline and 0.9% saline/0.05% NP-40. Autoradiography was performed after the last wash.

Blocking of a Monoclonal Antihuman μ (clone 1D12) with Antiidiotype Antibody

Further definition of the fine epitope specificity of the anti-idiotype antibodies was determined by their ability to compete for binding with a murine IgG1 MoAbs specific for human μ heavy chain, to the appropriate idiotype protein. Tumor cells stained by directly fluoresceinated 1D12 antibody in the presence or absence of anti-idiotype antibody were analyzed by flow cytometry and their mean fluorescences were compared. Tumor cells frozen in 10% dimethyl sulfoxide (DMSO) were thawed and viable cells were isolated using Ficoll-Hypaque density gradient centrifugation.

Twenty-minute incubations of 10^6 cells with 100 μL of 10 μg/mL of first stage antibody diluted in PBS/BSA/0.01% sodium azide were performed at 0 °C. The cells were washed twice with cold PBS/BSA/azide. The final antibody, 1D12FITC, was added to the cells which were incubated on ice, washed, and then fixed in 10% paraformaldehyde in PBS. Twenty thousand cells were counted in the flow cytometer and their mean fluorescence in linear units was determined.[44] The percent blocking was determined using the following equation:

$$\% \text{ blocking} = 1 - \frac{(\text{mean F anti-id} + \text{1D12FITC} - \text{background F})}{(\text{mean F irrelevant MoAb} + \text{1D12FITC} - \text{background F})} \times 100$$

Modulation of Surface Idiotypic Immunoglobulin

Aliquots of single cell suspensions of frozen biopsy specimens were thawed and viable mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. In order to induce modulation, aliquots of the tumor cell suspension were incubated with MoAbs in 5% CO_2 at 37 °C for varying periods of time. Cultures were started at different times to allow simultaneous staining of cells incubated for 0, 1, and 24 hours. After incubation, the cells were washed extensively with PBS/BSA and then stained for flow cytometry as described above. First stage antibodies used in the staining included an appropriate irrelevant MoAb, the appropriate anti-idiotype MoAb, and 1D12 MoAb. Binding of MoAb to the cells was detected with fluoresceinated goat anti-mouse IgG. The mean linear fluorescence was determined using a FACS IV (Becton Dickinson, Mountain View, Calif).[40] The percent modulation observed for a particular time and MoAb concentration was determined as follows:

$$\% \text{ modulation} = 1 - \frac{(\text{mean F modulated tumor}) - (\text{mean F tumor} + \text{second step})}{(\text{mean F unmodulated tumor}) - (\text{mean F tumor} + \text{second step})} \times 100$$

Tumor Cell Proliferation Assays

Two hundred thousand viable cells obtained from either a pretherapy tumor biopsy or peripheral blood mononuclear cells, if more than 90% were idiotype positive, were added to a 96-well microtiter plate in 200 μL of complete medium supplemented with sodium pyruvate (1 mmol/L) and 2-mercaptoethanol (10^{-4} mol/L). One half of the wells also contained 1 μg/mL phytohemagglutinin (PHA) (Wellcome, Greenville, NC) and phorbol myristic acetate 10 ng/mL (PMA) (Sigma Chemical Co, St. Louis, MO). To triplicates the appropriate anti-idiotype antibody, 1D12 (anti μ) or an isotype matched control antibody were added at a concentration of 10 μg/mL. The plates were incubated in 5% CO_2 at 37 °C for 2, 4, or 6 days and then pulsed with ^{3}H-methylthymidine at a concentration of 1 μCi per well. The cells were harvested and counted as previously described.[21]

RESULTS

Murine Antibody Isotype

The isotypes of the MoAbs used to treat the eleven patients are listed in Table 1. Strict criteria were used to define the specificity of each anti-idiotype antibody, and antibodies only of the IgG class were selected. However, no selection was imposed for IgG subclasses. In most instances the first hybridoma clone obtained with the appropriate specificity and stability was chosen for the therapeutic trial. The higher frequency of IgG1 anti-idiotype MoAbs reflects their greater frequency among all the hybridomas generated. Six patients received IgG1, two IgG2a, two IgG2b, and one received three antibodies, one of each isotype. There was no correlation in this study between the IgG subclass of the antibody and the occurrence of a tumor response. The only patient to achieve complete elimination of tumor received an IgG2b antibody; the next most dramatic responder was treated with an IgG2a. However, four of six patients infused with IgG1 anti-idiotype MoAb experienced objective

Table 1. Immunochemistry

Patient	PK	FS	BL	RD	BJ	CJ	CP	CG	TG	KL	PE
Treatment order	01	02	03	04	05	06	07	08	09	10	11
Response*	100	90	0	60	10	55	0	45	0	NE	90
Idiotype											
Phenotype	μλ	μκ	μκ	μλ	μκ	μκ	μλ	μκ	μκ	μλ	μκ
Density†	219	1479	389	224	ND	370	790	ND	186	ND	ND
Antibody	γ2b	γ1	γ1	γ1	γ2b	γ1	γ1	γ1	γ2a	γ1, γ2a, γ2b	γ2a
Epitope											
1D12 Block‡	15	77	9	62	ND	69	15	ND	81	10, 62, 72	ND
Chain§	HL	HL‖	H	HL	H	H	–¶	ND	HL	ND	HL
Affinity											
Rank	4	5	2	8		6	7		3		1
Plate#	230	64	>500	ND	ND	48	45	ND	410	ND	ND
Dilution#	100	41	530	<1	ND	34	29	ND	ND	38, 220, 36	1000

Abbreviations: ND, not determined; NE, not evaluable.

*Percent reduction in overall measurable tumor.

†Estimate of surface immunoglobulin per cell expressed as mean linear fluorescence of 2 × 10^5 tumor cells stained with 1D12-FITC.

‡Expressed as percent of maximal blocking of 1D12 by itself.

§Reactivity of antiidiotype antibody with idiotype protein on Western blot; H, heavy chain; HL, heavy and light chain together.

‖SDS-sensitive idiotope, renaturable.

¶SDS-sensitive idiotope, not renaturable.

#Binding constant × 10^7.

responses. Two other evaluable patients treated with IgG2 antibodies had no response.

Idiotypic Protein

All tumors described in this study expressed IgM on their cell surface. Four expressed λ light chain and seven expressed κ light chain. Six patients (one λ, five κ) secreted IgM which could be detected in the serum in concentrations of 0.1 to 400 μg/mL.[3] This secreted idiotypic protein was an IgM pentamer as determined by gel electrophoresis of samples isolated from the serum of two patients and from supernatant from "rescue hybrids" of the tumors of three others[34] (unpublished data). Although the presence of free idiotype protein in the serum could block binding of anti-idiotype to cellular idiotype, it did not preclude a favorable therapeutic result if a dose of anti-idiotype MoAb sufficient to achieve extravascular penetration was infused.

Estimation of the amount of surface IgM per tumor cell, revealed a sevenfold difference between the highest and lowest of the tumors tested (Table 1). The tumor cells from patient FS, with prolymphocytic leukemia, had nearly twice as much surface Ig as the next highest expressing tumor. Cells of the majority of patients clustered in the lower range, and there was no correlation between surface Ig expression and clinical response.

Epitope

The surface IgM of each individual tumor has a unique variable region with its own idiotypic determinants, but the majority of the molecule is made up of constant protein sequences shared by them all. The anti-idiotype MoAbs were by definition specific for determinants dependent upon the variable regions of the heavy and/or light chains of the tumor specific immunoglobulin. This epitope could be further defined by several methods. A particular MoAb, 1D12, which is specific for the human μ heavy chain and presumably reactive with a determinant in the constant region proximate to the variable region, could be significantly blocked by six of the ten anti-idiotype antibodies that were tested (Table 1). It is unclear whether the anti-idiotype antibodies that block sterically hinder the binding of 1D12 or if their binding produces changes in the tertiary structure which alter the 1D12 binding site. Different anti-idiotype antibodies against the same IgM molecule can differ in their effects on 1D12 antibody binding presumably because they bind to different sites (idiotopes) (Fig 1).

Immunoblots of reduced and unreduced idiotype protein provided additional information about the idiotypic determinant recognized by each antibody (Table 1). This technique can distinguish between antibodies which bind to an idiotope located on the isolated heavy or light chain, separated by gel electrophoresis after reduction, from those that require the intact immunoglobulin. Examples of both situations are displayed in Fig 2. In two cases, the electrophoresis procedure produced changes in the conformation of the idiotype of the unreduced molecule which prevented binding by the antiidiotype antibody.[41] The sodium dodecyl sulfate used in the electrophoresis was believed to be the factor

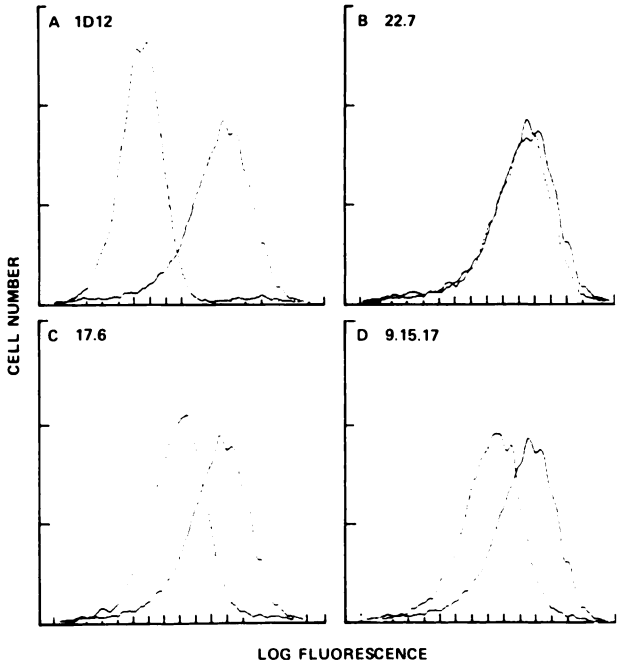

Fig 1. Anti-idiotype antibody blocking of 1D12. Histograms show the logarithmic fluorescence intensity after staining of a cell line derived from the tumor of patient KL which expressed idiotype-positive surface IgM. The cells in each histogram were stained with 1D12 anti-μ MoAb conjugated to fluorescein isothiocyanate (1D12-FITC) as a second step. The solid line in each panel represents initial incubation of the cells with a nonbinding isotype control antibody, showing maximal staining with the 1D12-FITC. The dashed line represents cells incubated previously with unconjugated antibody, (A) 1D12, (B) 22.7 anti-idiotype, (C) 17.6 anti-idiotype, and (D) 9.15.17 anti-idiotype. Displacement of the curve to the left represents the relative ability of the first step antibody to block the binding of 1D12-FITC to its epitope.

responsible and after washing with urea and NP-40 the binding site was restored in one of these two cases.[42] Of eight idiotypic proteins evaluable by this method, five reacted only with the assembled heavy-light chain molecule and three reacted with both the isolated heavy chain and the intact antibody. None of the anti-idiotype MoAbs reacted with the free light chain.

The ability to block 1D12 and the chain reactivity on Western blot analysis did not correlate with each other and appear to evaluate different facets of idiotypic fine specificity. Blocking of the 1D12 binding site did not correlate with clinical outcome. However, the data do suggest that reactivity with an HL combinatorial determinant may be important, that is, 4/5 responders *v* 1/2 nonresponders had HL reactivity.

Binding Constants

Another potentially important feature of the anti-idiotypic antibodies is their affinity or avidity. Determination of affinity requires accurate measurements of the concentrations of free and antibody-bound antigen under various conditions. In this case, both antigen and antibody are large proteins and complexes cannot be easily separated from free

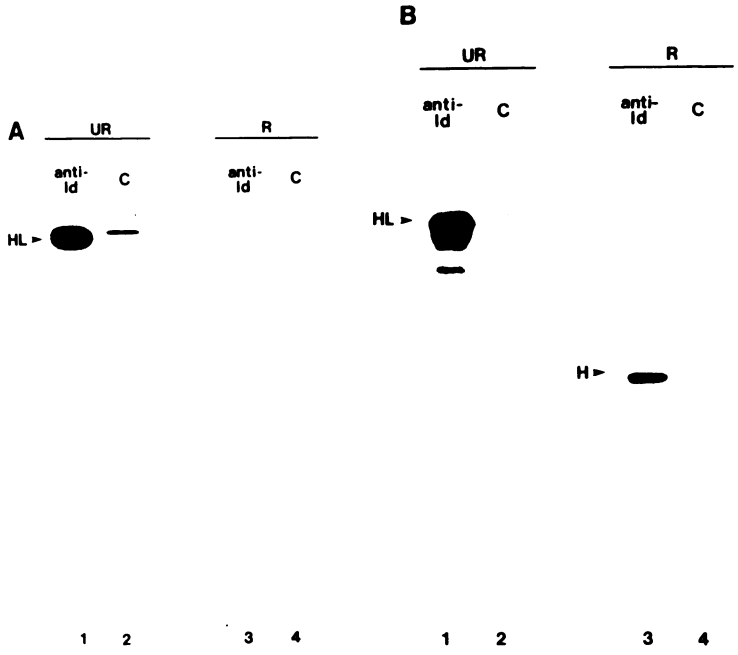

Fig 2. Immunoblotting of idiotype proteins with anti-idiotype antibodies. Idiotype protein was subjected to SDS-PAGE under reducing lanes 1 and 2 (R) and nonreducing lanes 3 and 4 (UR) conditions and electroblotted onto nitrocellulose membranes. Purified idiotype from patient PK (panel A) and CJ (panel B) were probed with the appropriate anti-idiotype (anti-id) or isotype matched control (C) antibodies and binding was detected with ^{125}I-goat anti-mouse Ig. Anti-idiotype for patient PK reacted with only the combined heavy and light chain and not with the isolated heavy or light chain. Anti-idiotype for patient CJ reacted with both isolated heavy chain and the intact molecule.

antigen. Two relatively independent methods were used to determine the binding constants of the anti-idiotypic MoAbs for which purified idiotype was available (Table 1). Both methods partition unbound antibody from bound antibody by fixing the immune complexes to a solid phase. In the equilibrium plate method the concentrations of bound and free anti-idiotype antibody are determined after interacting with the idiotype which was initially fixed to a plastic plate. The soluble complex method measures the amount of soluble complex formed by a fixed ratio of antibody and antigen at different total protein concentrations. These independent methods are clearly in agreement regarding the rank order of avidity of the MoAbs studied and remarkably close in their absolute values. A thousandfold difference existed between the most avid (PE) and the least avid (RD) antibodies. The highest and lowest values, however, were outside the most accurate quantitative range of both assays and the numerical values assigned to these two cases are less precise than the others. The rank order of these antibodies, however, is clear, and there was no correlation between this feature of the antibody and clinical response.

Modulation

Surface immunoglobulin, like many cell surface proteins, can move within the plane of the membrane and when cross-linked by multivalent antibody may be internalized causing the disappearance (modulation) of the surface antigen. The anti-idiotypic MoAbs caused a variable degree of modulation of cell surface idiotypic immunoglobulin both in vitro and in vivo. Fig 3 demonstrates differences in the modulability by their respective anti-idiotypic antibody of the tumors tested. The time dependence of modulation can be appreciated, as all tumors demonstrate increased modulation between one and 24 hours of incubation. Considerable variation existed among the tumors as to the maximal modulation which could be induced. CP exhibited no modulation at the 1 μg/mL concentration of anti-idiotypic MoAb and significantly less than the others at the higher concentration. Others were able to modulate 50% to 90% over 24 hours. Tumor cells of patient CG underwent extensive modulation in vivo as demonstrated in a lymph node specimen removed during treatment with anti-idiotypic MoAb (data

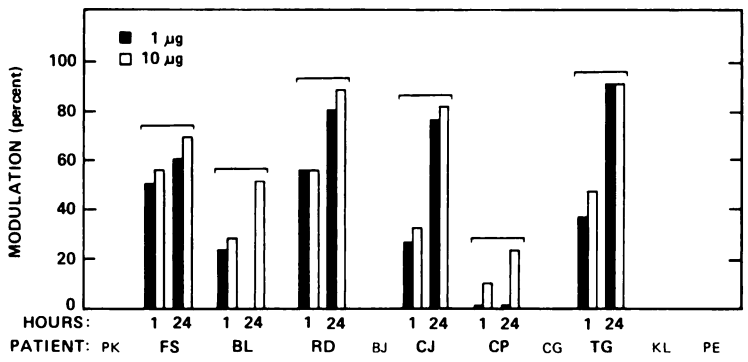

Fig 3. Modulation of surface immunoglobulin by anti-idiotype antibody. The tumor cells evaluated in Fig 3 were incubated for varying times with 1 or 10 μg/mL of anti-idiotype antibody. After staining with FITC-labeled goat anti-mouse Ig, the mean fluorescence of the cells was determined and related to that for cells incubated with a nonbinding control. Complete disappearance of surface immunoglobulin is equivalent to 100% modulation. The analysis was not performed on patients PK, BJ, CG, KL, and PE.

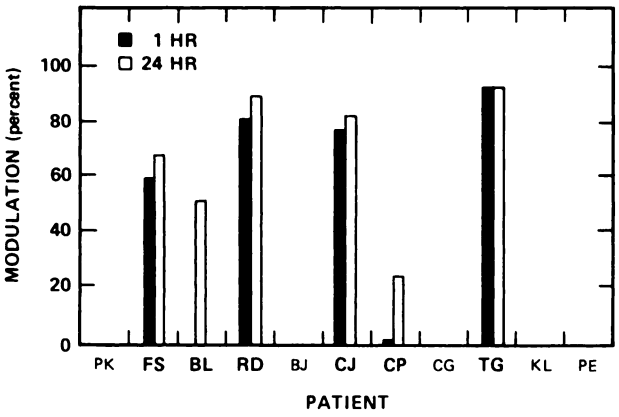

Fig 4. Modulation of surface immunoglobulin by 1D12 antibody. The patient's tumor cells were incubated for varying times with 1D12 (anti-μ chain MoAb) at a concentration of 10 μg/mL. The percent modulation was determined as described in Fig 3.

not shown). Modulation appeared to be a function of both the biologic behavior of the tumor cell and the MoAb used. Modulation induced by the anti-μ MoAb 1D12 (Fig 4) was similar to that induced by the anti-idiotype MoAb, however, the rate at which it occurred and the absolute degree of surface antigen decrement varied. Avidity, epitopic specificity, and clinical response did not correlate with in vitro modulation.

Effects of MoAb on In Vitro Proliferation of Tumor

In order to investigate whether the anti-idiotypic MoAbs had a direct effect upon their respective target tumor cells, we cultured the cells in vitro with the anti-idiotype or with the anti-μ 1D12 antibodies in the presence or absence of mitogens PHA and PMA for up to seven days. The results are displayed in Table 2. The addition of the mitogens PMA and PHA increased the baseline proliferation of the cells by a factor of three to ten (Table 2A), but the effects of the antibodies were the same whether or not the mitogens were present (Table 2B). The cells incubated without antibody maintained a stable proliferative state over the seven-day period. Three of the tumors were unaffected by the addition of either the anti-idiotype or the anti-μ antibody. Two other tumors were mildly affected (TG was suppressed; BL was stimulated) by both their respective anti-idiotypic antibody and by anti-μ. Neither of these patients experienced any change in their tumors upon treatment with their anti-idiotype antibodies. By contrast, tumor cells from patient FS were markedly suppressed by both anti-idiotype and anti-μ MoAb. This patient, with prolymphocytic leukemia, had a dramatic and sudden response to anti-idiotype antibody therapy, in contrast to the rather gradual responses observed in other responding patients.[3] The rapid tempo of the clinical response in patient FS and the unusual susceptibility of his tumor cells to a direct antiproliferative effect by anti-Ig antibodies in vitro suggest the mechanism for his response was different from that for the other patients.

Host Factors

The various tissues obtained from the patients were analyzed by immunofluorescence and flow cytometry of cells in suspension and by immunoperoxidase staining of frozen tissue sections. All specimens contained numerous infiltrating normal cells. As previously reported,[15] the normal host cells infiltrating the tumor were composed primarily of T lymphocytes. These T cells were predominantly of the Leu 3, Leu 4 (T4, T3) positive (helper) phenotype (Table 3). Smaller numbers of Leu 2, Leu 4 (T8, T3) positive (cytotoxic, suppressor) T cells were also present, as were a small,

Table 2. Proliferation Assays

	PK	FS	BL	RD	BJ	CJ	CP	CG	TG	KL	PE
A.											
CONTROL MoAb*											
Day 3	ND	145	5.5	20	ND	2	21	ND	32	ND	ND
−Mit†	ND	95	0.7	3	ND	0.8	4	ND	4	ND	ND
Day 5	ND	55	7	26	ND	2.5	13	ND	31	ND	ND
−Mit†	ND	10	0.5	2.5	ND	1	5	ND	3	ND	ND
Day 7	ND	50	10	29	ND	3	12	ND	30	ND	ND
−Mit†	ND	4	0.5	2.5	ND	1	3	ND	3	ND	ND
B.											
ANTIIDIOTYPE MoAb‡											
Day 3	ND	79	145	100	ND	100	100	ND	74	ND	ND
Day 5	ND	63	157	100	ND	100	100	ND	73	ND	ND
Day 7	ND	14	149	100	ND	100	100	ND	74	ND	ND
ANTI-MoAb‡											
Day 3	ND	62	120	100	ND	100	100	ND	75	ND	ND
Day 5	ND	45	125	100	ND	100	100	ND	75	ND	ND
Day 7	ND	14	112	100	ND	100	100	ND	76	ND	ND

ND, not determined.

*Proliferation of tumor cells as measured by ^{3}H-thymidine incorporation in counts per minute × 10^{-3}.

†Wells without mitogens.

‡Reported as percent of the ^{3}H-thymidine incorporation seen with control MoAb (Table 2A)

Table 3. Pretherapy Biopsy Specimens Analysis with Monoclonal Antibodies*

Patient	Total Pre-Therapy Biopsies†	Diagnosis‡	Tissue§	Leu-4	Leu-3	Leu-2	Leu-7	Tumor Cells	Response‖
PK	2	N/DLPD	LN	51	36	7	7	30	100
FS	1	PLL	BM	8	11	1	0.5	83	90
BL	1	N/DLPD	LN	15	13	6	2	79	0
RD	1	DLPD/LCL	LN	31	14	8	2	80	60
BJ	1	NML	LN	16	15	5	2	80	10
CJ	2	NLPD	LN	34	20	8	4	88	55
CP	2	NLPD	LN	19	16	4	2	86	0
CG	2	NML	Spleen	23	24	3	2.7	79	45
TG	1	NLPD	Spleen	7	7	1	0.4	90	0
KL	2	DLCL	LN	6.6	10	1.7	ND	80.7	NE
PE	3	N/DPLD	LN	77	70	9	5.1	26.1	90

Abbreviations: ND, not determined; NE, not evaluable.

*Reported as percent of total cells positive for each antibody as determined by flow cytometry, except for patient PK and all Leu 7 data which was determined from immunoperoxidase-stained frozen sections.

†Numbers reported are representative of all pretherapy biopsies.

‡NLPD: nodular lymphocytic poorly differentiated, DLPD: diffuse lymphocytic poorly differentiated, PLL: prolymphocytic leukemia, LCL: large cell lymphoma, NML: nodular mixed lymphoma.

§LN: lymph node, BM: bone marrow.

‖Percent reduction in overall measurable tumor.

variable number of Leu 7 positive (NK/K) cells. Leu 7 positive cells were large and localized to follicles in patients with follicular histology. In patients with follicular diseases the host infiltrate cells were localized predominantly in the interfollicular areas.

Interestingly, we observed that ratios of malignant B cells to nontumor T cells existed which were characteristic for each patient. For instance, in one patient, pretherapy specimens were obtained from three different tissue sites (lymph node, skin, and bone marrow) at two separate time points. All three biopsies were found to have maintained a similar ratio of tumor cells to host infiltrate (Fig 5).

There was a correlation between the number of nontumor T cells present in biopsy specimens prior to anti-idiotype therapy and the response to that therapy (Fig 6). This correlation was especially striking in view of the failure of the many other parameters of the therapeutic MoAb, the tumor cell, and the host mentioned above to predict response to anti-idiotype therapy. Of the two patients with tumors containing 56% to 80% Leu 4 positive cells in the biopsy, the disease in one completely remitted with anti-idiotype MoAb and that in the other showed an impressive response. The difference in the proportion of T cells in these two patients compared to the other patients was statistically significant ($P < .001$). The patients with lesser responses had between 23% and 34% Leu 4+ cells in their pre-MoAb therapy tissue biopsies. The patients who had no tumor response all had less than 16% T cells in their specimens. This evaluation excludes patient FS, whose disease was a leukemia rather than a lymphoma and whose tumor response appeared to be mediated by a direct antiproliferative effect.

DISCUSSION

A series of patients with B cell malignancy has been treated with MoAbs directed against an idiotypic determinant of the immunoglobulin molecule present on the surface of their tumor cells. A spectrum of clinical outcomes has been observed, ranging from complete and durable remission to no effect.[1,3] Some of the factors that influence the therapeutic outcome have been previously identified and include the presence in the serum of idiotype protein, the occurrence of an immune response against the foreign MoAb, and the existence within the tumor population of cells with mutations of the idiotype.[3,13,14] In view of these obstacles, it is remarkable that clinically significant tumor regressions have occurred in over half of the patients who have been studied. In fact, the emergence of tumor cells with idiotype mutations during therapy with an anti-idiotype antibody implies that the antibody exerted a strong selective force against the idiotype-positive tumor cell population in these cases.

In the current report we have presented a series of studies to elucidate the mechanism of the antitumor effect of the anti-idiotype antibodies. Since each patient, tumor and antibody were unique and only limited amounts of pathologic material were available from some of the patients, exhaustive studies were difficult to perform. Nevertheless, we attempted to examine the relative roles of the antibody—its class, affinity, and epitope; the tumor—its responsiveness to direct effects of anti-idiotype antibody and the surface density and modulability of its Ig molecule; and the host—the nature and composition of the cellular infiltrate within the tumor tissue.

The known mechanisms of in vitro antibody mediated cytotoxicity include complement fixation and antibody-dependent cell-mediated cytotoxicity (ADCC). Both of these functions are highly dependent on antibody class as well as the availability of the effector molecules and/or cells. Kipps et al have recently documented the superiority of murine IgG2a over IgG2b and IgG1 antibodies in performing ADCC in vitro with human effector cells,[43] and numerous studies in animal models have likewise suggested a superiority of IgG2a antibodies for in vivo antitumor effects.[44-46]

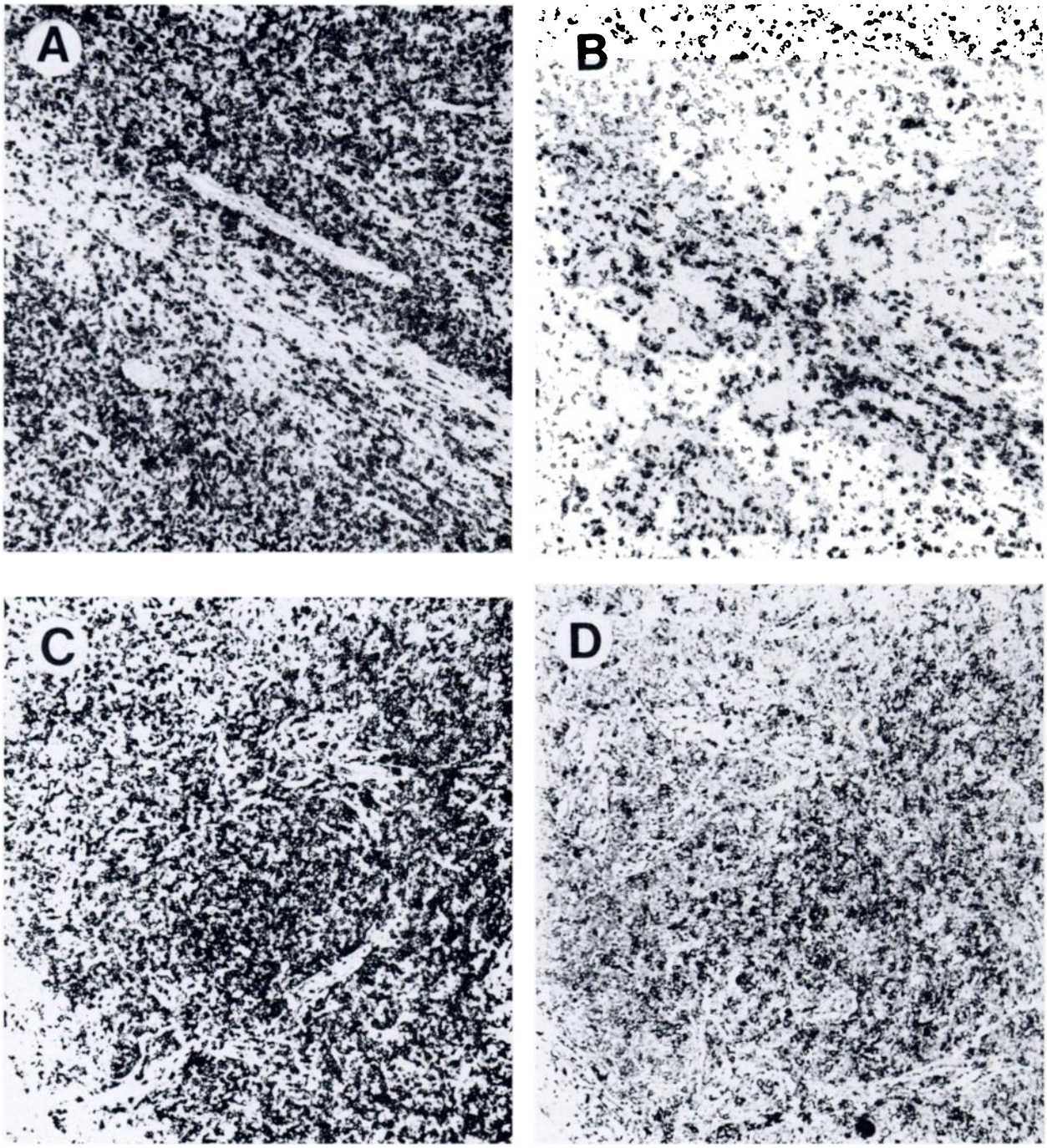

Fig 5. Relative numbers of infiltrating host T Lymphocytes and malignant B cells are demonstrated by immunoperoxidase staining of frozen sections of pretherapy tumor specimens from patient PE. Simultaneous biopsy of lymph node (A,B) and subcutaneous mass (C,D) were performed in April 1982. A bone marrow biopsy (E,F) was performed in August 1984 immediately prior to treatment with anti-idiotype antibody. Frozen sections of each biopsy specimen were incubated with anti-Leu 4, which binds to mature T lymphocytes, (A,C,E), or an antibody specific for a B lymphoid cell surface marker (B-anti-μ, D-anti-B1, F-anti-μ). Cells binding antibody were detected with an immunoperoxidase method and the specimen was counterstained with methylene blue. The large excess of host T lymphocytes to tumor cells is maintained over time and in various tissues involved with tumor.

There was no clear relationship in our clinical trial between antibody class and outcome. Although the most dramatic responses occurred with antibodies of the IgG2 class (one IgG2a and one IgG2b), responses occurred with antibodies of the IgG1 class as well. In addition, we have not observed a correlation between changes in serum complement levels and clinical outcome. The observations tend to argue against an important role for a direct cytotoxic effect of anti-idiotype antibodies, although they do not rule out such a mechanism.

Whatever the mechanism of antibody effect, it seemed

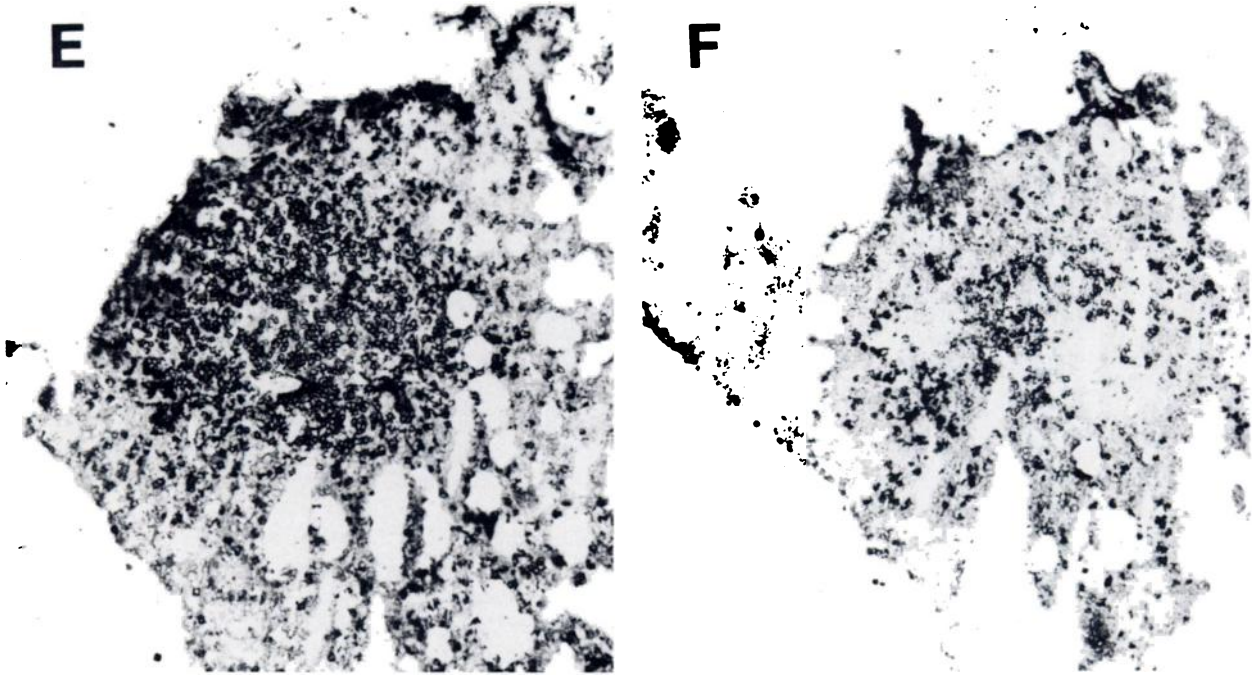

Fig 5. (Cont'd).

likely that the avidity of interaction between antibody and idiotype target should be important. Two different methods were used in this study to estimate antibody avidity. The results were in close agreement, and certainly there was concordance in the rank order of the different antibody-idiotype combinations. Over the range of avidity covered by these particular antibodies no correlation existed with clinical outcome. Apparently the methods that were used to select MoAbs resulted in antibodies of sufficiently high avidity to produce in vivo effects.

Although idiotypic determinants are located somewhere within the variable regions of the immunoglobulin heavy and light chains, different anti-idiotypic antibodies can recognize different parts of the variable region structure. For instance, in cases of myeloma proteins which bind known antigens, some anti-idiotypic antibodies inhibit antigen binding and some do not.[47-49] If anti-idiotype antibodies exert a direct regulatory effect on the B cell which bears the relevant target molecule on its surface, the exact portion of the V region to which they bind may be of critical importance in signal transduction. Since in B cell lymphomas no antigen is known to bind to the cell surface idiotype, we used two alternative approaches to investigate the idiotypic determinants. A MoAb reactive with the μ heavy chain was blocked from binding to its respective epitope by certain anti-idiotype antibodies. Evidently, this anti-μ antibody reacted with a site in the C_μ region near the V-C junction. We could then infer that some of our anti-idiotype antibodies reacted with a site nearby. Some idiotypic determinants are created by a combination of heavy and light chain structures, and others are dependent only upon one of the chains. We tested the antibodies which were used in the therapeutic trials for their ability to react with isolated chains and found that several of them reacted with determinants on the heavy chains. Steric hindrance of an anti-μ chain antibody showed no correlation with outcome whereas reactivity with an HL combinatorial determinant did show some correlation with clinical effectiveness in vivo.

Direct effects of anti-idiotype or anti-Ig antibodies on normal and neoplastic B cells have been demonstrated in a number of in vitro systems.[16-18,23,24,50,51] These effects have included stimulation of proliferation, inhibition of proliferation, and induction of receptors for BCGF and IL2. The differences seem to depend upon the in vitro system, the particular anti-Ig reagent and the state of differentiation of

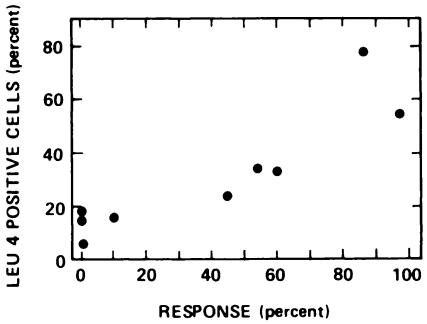

Fig 6. Impact of T cells on response to anti-idiotype therapy. The percent of Leu 4 or Leu 1 positive cells, as determined by flow cytometry in the pretherapy biopsy of patients is plotted on the vertical axis and the percent response measured by lymphangiogram or computerized axial tomography is plotted on the horizontal axis. The percent of T cells is the average measured in all 15 pretherapy biopsies of the nine patients eligible for this analysis. The two patients with the best responses were biopsied two and three times prior to anti-idiotype therapy.

the B cell target. The antiproliferative effect of anti-Ig on normal B cells can be prevented by prior treatment with mitogens.[17] It was thus important to investigate the direct effects of the anti-idiotype antibodies which were used for therapy on their respective tumor target cells. We found that the antibody used in one patient (FS) had a profound inhibitory effect on in vitro tumor cell proliferation. The antibody used in patient BL had a slight stimulatory effect, and that used in patient TG had a mild inhibitory effect on tumor cell proliferation. Those used on the other three patients tested had no measurable effect in vitro. The effects of antibody on the cells were unchanged by concomitant mitogen stimulation. The anti-idiotype antibodies used for patients BL and TG had no in vivo effect on their tumors, either positive or negative. The kinetics of the tumor response in patient FS were consistent with a direct antiproliferative effect, since there was an acute reduction of tumor cells in the blood and bone marrow. Patient FS was different from the other patients in our series in that he had prolymphocytic leukemia, whereas the others had lymphomas. The FS tumor cells were larger and expressed markedly more surface Ig than the others. Gordon et al found EBV-transformed lymphoblastoid B cells to be inhibited by anti-Ig antibodies.[18] Prolymphocytic leukemia cells might be "fixed" in a normal differentiation state susceptible to regulation by anti-idiotype antibody.

Modulation, or "capping," of the surface antigen target by MoAb had important implications in systems in which ADCC or antiproliferative effects of antibodies were studied.[16,23,52] Univalent (nonmodulating) antibodies have been shown to be more cytolytic both in vivo and in vitro than their bivalent counterparts which can cross-link surface antigen and produce modulation.[52] Capping of surface Ig is required for signal transduction in the antiproliferative and tolerogenic effects produced by anti-Ig antibody on neonatal murine B cells.[16] Differences in the capacity for modulation were observed among the patients' tumor cells in this study which might have influenced response to therapy. However, Schroff et al demonstrated the enhancement of modulation by Fc receptor-bearing monocytes.[53] The in vitro modulation of the individual tumors observed in this study may have been influenced by the presence of variable numbers of macrophages/monocytes in each suspension. Similarly, the availability of Fc receptor-bearing cells during in vivo therapy might change the tumors' ability to modulate in the presence of anti-idiotype antibody. Within these limitations, no correlation existed between in vitro modulability and clinical outcome.

In some MoAb clinical trials, the suggestion has been made that an immune response by the patients against the murine immunoglobulin was beneficial.[54] Theories have been advanced about second and third order immune responses involving idiotype—anti-idiotype networks, ultimately leading to an immune attack by the host against the tumor.[54] In our trial, an immune response against the murine immunoglobulin was documented in several of the patients, and in each case a portion of the response was indeed directed against the idiotypic determinant of the mouse monoclonal antibody.[3] However, no support can be found in our data for a role of the anti-mouse immunoglobulin immune response in the antitumor effect. The two most dramatic tumor regressions occurred in patients who did not make an immune response. Furthermore, in those patients who did make an immune response, no additional tumor regression was ever noted after that point in time.

Although an immune response by the host against the mouse MoAb seems unlikely to be therapeutic, an immune response against the tumor is a possible mechanism. However, this immune response seems to exist in the host prior to the introduction of the MoAb.[14,15,55-57] In our study, there was a good correlation between clinical outcome and the number of T cells present in the pretherapy tumor tissue. In the two best responding cases the T cells actually outnumbered the tumor cells. Analysis of the cellular infiltrate showed the majority of the T cells to be of the helper/inducer (Leu 3, OKT4) phenotype in each case. Also present in this cell population were Leu 7 positive (putative NK) cells. One view would then be that the MoAb amplified or augmented an ongoing cell-mediated cytotoxic response by the host against the tumor, tipping the balance back in favor of the host. The fact that the T cells were of the Leu 3 phenotype is not inconsistent with this hypothesis, since Leu 3 positive, T killer cells that are restricted in their target cell recognition by the class II MHC structures are well described.[58] An alternative view is that the interaction between host T cells and tumor cells is a positive or trophic one. Activated T cells are a rich source of growth stimulatory factors for B cells. The fact that T cells were found associated with the tumor in relatively fixed proportions in various sites throughout the body is equally consistent with a positive as well as a negative interaction. In this view, the MoAb would be interrupting or somehow interfering with the trophic effect of the T cells on the growth of the tumor. Whether either of these mechanisms of anti-idiotype antibody effect are operative can only be the subject of speculation at the present time. Clearly, more observations must be made as more patients are treated. Also, it will be extremely important to isolate the T cells from the tumor specimens, to measure their effects on the tumor cells, and to determine whether anti-idiotype antibodies alter these effects.

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