Documentation of Idiotypic Cascade after Lym-1 Radioimmunotherapy in a Patient with Non-Hodgkin's Lymphoma: Basis for Extended Survival?[1]

Bonnie M. Bradt, Sally J. DeNardo, Gary R. Mirick, and Gerald L. DeNardo[2]
University of California Davis Medical Center, Sacramento, California 95816

Abstract

Purpose: **The purpose of this study was to examine idiotypic cascade mechanisms in the plasma of a prolonged survivor patient with aggressive non-Hodgkin's lymphoma (NHL). It is a follow-up to previously published seminal studies by this laboratory showing survival benefit associated with radioimmunotherapy in NHL patients. Immunoglobulin from the patient's plasma was purified, characterized, and shown to possess the activities expected of idiotypic antibodies.**

Experimental Design: **Plasma from a NHL patient treated with Lym-1 was precipitated with ammonium sulfate and octanoic acid, followed by immunoadsorbant chromatography with solid phase Lym-1 monoclonal antibody to purify Ab2. The last purification step involved the binding of Ab3 to glutaraldehyde-fixed Raji cells, followed by acid elution of Ab3. Proteins were quantified and characterized. Antibody-dependent cellular cytotoxicity activity was determined using a standard ^{51}Cr release assay.**

Results: **Purified immunoglobulin populations exhibited the characteristics of Ab2β and Ab3 antibodies. Both showed ability to compete with the binding of Lym-1 to its tumor cell target, and Ab3 showed ability to induce antibody-dependent cellular cytotoxicity.**

Conclusions: **This study offers direct evidence for initiation of a multilevel idiotypic cascade in a patient undergoing passive monoclonal antibody therapy for NHL. The patient's prolonged disease-free survival may, thus, be understood in the context of the generation of endogenous, self-perpetuating tumor-specific antibodies.**

Introduction

Successful results have been achieved using radiolabeled (1–5) and nonradiolabeled (6–8) antibodies for cancer therapy. The therapeutic success can be, in part, attributed to the phenomenon of passive immunotherapy, which either uses the biological functions of injected MAbs[3] against tumor cells or uses them as a delivery vehicle for radionuclides or toxins (9). Alternatively, active immunotherapy or the use of the patient's immune system to elicit antitumor activity can be an attractive approach. The activity can be demonstrated through antibody generation (vaccination) or by the activation of cellular-based tumor cytotoxicity (9–11). This active approach to immunotherapy is more durable and multifaceted in nature than that of passive immunotherapy.

Mouse MAbs used for passive immunotherapy have been observed to elicit an immune response in the human recipient (6, 8, 12), against the constant and/or the variable region of the therapeutic MAb. These HAMAs can be, therefore, both anti-isotypic and anti-idiotypic in nature, depending on the target epitope. The frequency of HAMA response to a murine MAb is generally high among nonhematopoetic cancer patients. In malignancies of the hematopoetic system, the HAMA response is variable (12), possibly dependent on the state of the patient's immune system at the time of MAb administration.

The idiotypic network theory (13) predicts that the idiotopes of the initially injected MAb (Ab1) can elicit an anti-id response antibody (Ab2) and eventually an anti-anti-idiotypic (Ab3) response in the patient. Because certain subpopulations of the Ab2 response (Ab2β antibodies) contain a part of their variable region that physically mimics the original epitope on the tumor cell recognized by the Ab1, a portion of the Ab3 immune response mounted by the patient may be directed against the tumor cell antigen itself.

In a previous study (14), we described a patient with aggressive, chemotherapy-resistant NHL who achieved prolonged disease-free survival after treatment with mouse MAb Lym-1. Mechanisms implicating beneficial effects of the high HAMA levels in this patient, shown to be largely idiotypic in nature, were postulated. Recovery of plasma antibodies possessing the activity ascribed to members of the idiotypic cascade supports the hypothesis that this patient's prolonged survival was related to increased antitumor activity of her immune system, induced by the treatments with Lym-1 MAb and continuing well after Lym-1 passive immunotherapy was discontinued.

Materials and Methods

Patient Case Report. Because of short or incomplete remissions after standard therapy regimens, a rapidly progress-

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[2] To whom requests for reprints should be addressed, at Radiodiagnosis and Therapy, 1508 Alhambra Boulevard, #3100, Sacramento, CA 95816. Phone: (916) 734-3787; Fax: (916) 451-2857. E-mail: gldenardo@ucdavis.edu.

[3] The abbreviations used are: MAb, monoclonal antibody; NHL, non-Hodgkin's lymphoma; ADCC, antibody-dependent cellular cytotoxicity; HAMA, human antimouse immunoglobulin antibody; id, idiotypic antibody; SAS, saturated ammonium sulfate.

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ing scalp mass, and Ann Arbor stage 4 NHL, a 47-year-old female was referred for ^{131}I-Lym-1 therapy. Pathological examination of biopsy samples confirmed Lym-1-positive diffuse, small cleaved B-cell intermediate grade NHL. Imaging verified uptake of the MAb by the tumors, and the patient was given therapy. Within 1 month of initial therapy, the patient exhibited seroconversion against the Lym-1. Because the patient's response to therapy was excellent but incomplete, it was continued despite the HAMA seroconversion. Before each successive therapeutic dose, plasmaphoresis and additional Lym-1 preload were used to lower the patient's circulating HAMA levels. With each additional dose of Lym-1, HAMA titer increased. Therapy was discontinued after four therapy doses because the HAMA titers could not be reduced adequately. By this time, the scalp mass had resolved. The patient experienced only mild toxicities during therapy, which were responsive to administered steroids.

MAbs. Murine MAb Lym-1 (Damon Biotech, Inc., Needham Heights, MA), an IgG2a that is B lymphocyte specific and has high affinity for a membrane-associated antigen (a discontinuous epitope on the β chain of the HLA-DR10), is up-regulated on malignant B cells (15, 16). This hybridoma was generated by fusion of splenic lymphocytes from mice immunized with a nuclear fraction of Raji cells, a B-cell line originating from a Burkitt's lymphoma patient. Lym-1 antigen is not shed from the lymphoma cell surface or internalized, and it has a high surface density on malignant B cells (15).

Mouse L6 (Ref. 17; Oncogen, Seattle WA) is also an IgG2a MAb generated using non-small cell lung carcinoma as the immunogen. It recognizes a 202-amino acid, hydrophobic, and cysteine-rich membrane-associated polypeptide, which appears to exist in a multiprotein complex on the surface of many types of carcinoma cells. Mouse L6 is not reactive with the HLA-DR10 antigen and was used as the isotype-matched control antibody in assays described here.

Radiopharmaceutical. Lym-1 was radiolabeled by the chloramine-T method with ^{125}I for *in vitro* assays or ^{131}I for imaging and therapy. Details of preparation and administration have been published previously (14). High-performance liquid chromatography and cellulose acetate electrophoresis demonstrated that greater than 95% of the radioactivity was associated with monomeric antibody. A solid phase immunoreactivity assay using Raji cells showed at least 90% binding of the ^{131}I-Lym-1 relative to an ^{125}I-Lym-1 reference standard, and the final radiopharmaceuticals were pyrogen free by *Limulus* amebocyte lysate assay.

Cell Lines. Raji cells (human Burkitt's lymphoma) were purchased from American Type Culture Collection and maintained in RPMI 1640 with 10% FCS, 1% L-glutamine (200 mM), 1% sodium pyruvate (100 mM), 1% nonessential amino acids, and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere.

Isolation of Anti-idiotypic (Ab2) and Anti-anti-idiotypic (Ab3) Antibodies from Plasma. Plasma samples from the patient were first enriched in immunoglobulin purity through the use of a 50% SAS precipitation, resuspended to the original starting volume of plasma, and dialyzed in PBS. This was followed by an octanoic (caprylic) acid precipitation as described previously (18). Briefly, while stirring, 2 volumes of 60 mM sodium acetate buffer (pH 4.8) were added to the dialyzed SAS cut material. Caprylic acid was then added slowly at the rate of 0.7 ml per 10 ml of starting material. The mixture was incubated, stirring, for 30 min at room temperature and then centrifuged at 5000 × *g* for 10 min. The supernatant was dialyzed with PBS. A second 50% SAS precipitation provided the product in a concentrated form (approximately 10 mg/ml total protein concentration).

To isolate Ab2, immunoadsorbant beads were prepared by coupling purified MAbs to Aminolink beads (Pierce Chemical Co., Rockford, IL) using the manufacturer's protocol. One hundred milligrams of Lym-1 were coupled to a 10-ml settled bead volume, and 50 mg of L6 were coupled to 5 ml of beads. Immunoglobulin fractions from plasma samples, which had been shown previously to be positive for anti-Lym-1 antibodies by the HAMA ELISA, were sequentially adsorbed on the Lym-1 and the L6 resins to remove anti-id and anti-isotypic antibodies, respectively. Lym-1-specific anti-id antibodies ranged from 60–80% in the preparations before passage over the L6 column. Bound antibodies were eluted from the resins, after thorough washing, by 0.1 M glycine buffer (pH 2.5) and immediately neutralized with 1 M Tris buffer (pH 8). Eluted material was then dialyzed against PBS.

Isolation of Ab3 antibodies was accomplished using a process of immunoadsorption to glutaraldehyde-fixed Raji cells. The cells were prepared by initial washing into PBS and cooling on ice at a concentration of 5 × 10^7/ml. A solution of 0.5% gluteraldehyde in cold PBS was added to the cells with constant gentle swirling until a concentration of 1 × 10^6 cells/ml was reached. After incubation for 20 min on ice with occasional swirling, the cells were spun down and washed twice with PBS containing 1% BSA and then twice more with PBS with no additives. The fixed cells were stored in PBS containing 0.01% sodium azide as a preservative. Immunoglobulin fractions from the HAMA-positive plasma, after first being exposed to the Lym-1 resin to remove Ab2, were incubated, in a batchwise manner, with the fixed Raji cell pellet. After overnight incubation, with constant mixing, at 4°C, the fixed cells were pelleted, washed thoroughly with PBS, and eluted with 0.1 M glycine (pH 2.5), neutralized with 1 M Tris (pH 8), and dialyzed *versus* PBS. Plasma samples used for these purifications were pretherapy samples, carefully documented to contain no detectable circulating levels of Lym-1 (Ab1), which would otherwise be copurifying at this point, with the Ab3. Protein quantitation for all preparations was performed using the bicinchoninic acid protein assay kit (Pierce Chemical Co.). PAGE analysis and Western blotting were performed using precast 4–20% gradient Nu-PAGE gels from NOVEX (San Diego, CA), as per the manufacturer's directions.

ELISA for HAMA Quantitation. Titration of HAMA levels in human plasma were performed in an ELISA format. Ninety-six-well plates (PRO-BIND; Becton Dickinson, Lincoln Park, NJ) were coated with 2 μg (100 μl) of Lym-1 at pH 9 for 1 h at 37°C. Wells were blocked with PBS/5%BSA for 30 min. After this and subsequent incubations, wells were washed with PBS containing 0.1% Tween 20. Replicate 50-μl plasma samples in PBS/BSA (undiluted, 1:10 and 1:100) were incubated for 1 h at 37°C. Bound human IgG was detected using biotinylated mouse antihuman IgG (Sigma, St. Louis, MO), followed by streptavidin-horseradish peroxidase (Amersham, Arlington

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Heights, IL) and 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma). The reaction was stopped with 50 μl of 10% SDS and read at 405 nm in an ELISA reader (Dynatech, Chantilly, VA).

Competitive Inhibition RIA for Ab2 and Ab3. Inhibition of binding of Lym-1 antibody to the tumor antigen present on Raji cells was measured in a RIA using ^{125}I-Lym-1 (19). Briefly, a series of dilutions of excess purified Ab2 and Ab3 preparations were used as competitors for the binding of 10 ng of radiolabeled Lym-1 to 1 million Raji cells in a total volume of 150 μl. The mixture was incubated at 25°C for 1 h, then spun down. Cells and supernatant were counted, and percentage of binding was determined. The buffer used was PBS with 1% BSA.

ADCC Assay. Raji cells were labeled in 750 μl of complete RPMI containing 150 μCi of ^{51}Cr by incubation for 2 h at 37°C. Cells were washed in the same medium and suspended at a concentration of 5×10^4/ml. Labeled cells were added to individual wells of a 96-well microtiter plate together with 1:3 dilutions of crude immune plasma, normal plasma, or purified Ab3. Peripheral blood mononuclear cells isolated by Ficoll-Hypaque centrifugation from a volunteer donor were then added to the plate at the E:T ratio of 100:1. Plates were incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. The plate was then spun, and radioactivity released into the supernatant was measured (in cpm). The amount of spontaneous release was determined in control wells containing only labeled target cells. Maximum possible release was determined by lysing the cells with 1 N HCl. The percent of specific lysis was determined by the standard formula of corrected experimental lysis divided by corrected maximum lysis multiplied by 100.

Results

Enrichment of Immunoglobulin Populations. After the initial appearance of HAMA, the patient underwent plasmapheresis (3- to 4-liter exchange per day) before each of three Lym-1 administrations. Replacement fluids consisted of equal volumes of 5% albumin and normal saline. A fourth plasmapheresis was attempted but failed to reduce circulating HAMA adequately, thus no treatment was given. Plasmapheresis material was frozen at −20°C in aliquots and retained, offering the starting material for these purifications. HAMA assays were performed on the patient's plasma and on the material obtained by the plasmapheresis during the period of her active treatment (Fig. 1). Initial imaging and therapy doses were given in May and seroconversion occurred within one month. As can be seen, HAMA reached high titers after three therapy treatments, and before the fourth therapy, plasmapheresis and Lym-1 MAb preload failed to reduce her HAMA adequately. There was rapid clearance of the radiolabeled Lym-1 from her circulation during the fourth therapy and barely detectable tumor uptake. Therapy treatments were, therefore, discontinued. At this point, minimal disease was present because of the effectiveness of the earlier therapies (14).

For all plasmapheresis preparations, purification provided material that was 75–95% reactive with antihuman IgG in Western blot analysis (data not shown).

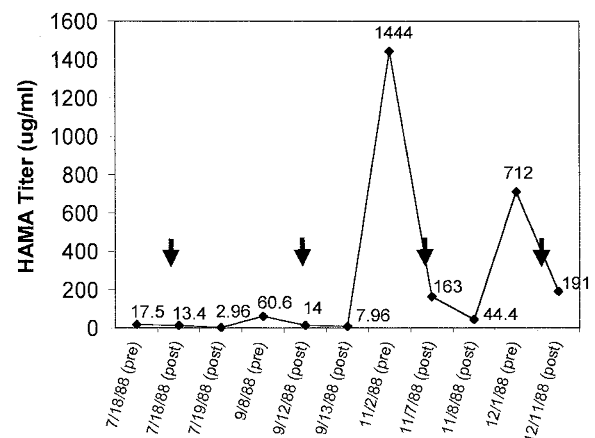

Fig. 1 HAMA titration was performed on the patient's plasma during the course of her treatment with Lym-1 RIT. Abscissa represents dates of sample collection subsequent to seroconversion. *Arrows* represent the dates of her therapies. The first therapy was administered in May. The patient was given a total, throughout the course of her therapy, of 300 mg of Lym-1. Plasmaphoresis was performed on four occasions before therapy to reduce her HAMA titers. All Ab2 and Ab3 preparations reported here derived from either the November or December plasmaphoresis.

Purification of Ab2. When starting with 2–2.5 grams of enriched immunoglobulin (from 500 ml of starting material), at least three sequential exposures to the Lym-1 immunobeads were necessary to recover the majority of anti-Lym-1 antibody. HAMA assays were performed at each step, both against Lym-1 antibody and against L6 isotype-matched control antibody. This comparative assay system allowed the determination of anti-isotype *versus* anti-id (Lym-1 specific) antibody in the preparations. Ab2 preparations were initially determined to contain approximately 60–80% Lym-1-specific anti-id activity, on a purified protein basis. This activity was similar to the percentage of specific anti-Lym-1 activity per total HAMA titered in the starting material. Anti-isotypic activity was removed on exposure of the material to L6-immunobeads. Representative purified pools of Ab2 on PAGE are shown in Fig. 2. Differences in yield were seen from different plasmaphoresis material, but activity and purity of the final preparations were similar.

Activity titers of the Ab2 preparations were determined by using them as competitors to inhibit the binding of radiolabeled Lym-1 to Raji cells. Fig. 3 shows the comparison of several Ab2 preparations to unlabeled Lym-1 itself, used as the blocking MAb. The activities of the antibody preparations compared favorably and were also similar in their ability to block this interaction to Lym-1.

Purification of Ab3. Preliminary experiments were performed using a RIA based on ^{125}I-Lym-1 and glutaraldehyde-fixed Raji cells (Fig. 4). These assays demonstrated that fixation of Raji cells occurred without loss of the HLA-DR10 (Lym-1 antigen) on the surface of the cells. RIA experiments showed that the level of binding of radiolabeled Lym-1 was not diminished after as many as four to five successive uses of the fixed cell pellet (data not shown).

PAGE of a representative preparation of Ab3 is shown in

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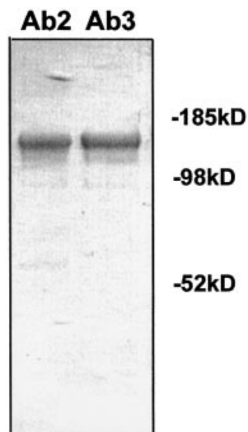

Fig. 2 SDS-PAGE gel depicts representative preparations of Ab2 and Ab3. Positions of molecular weight markers are indicated.

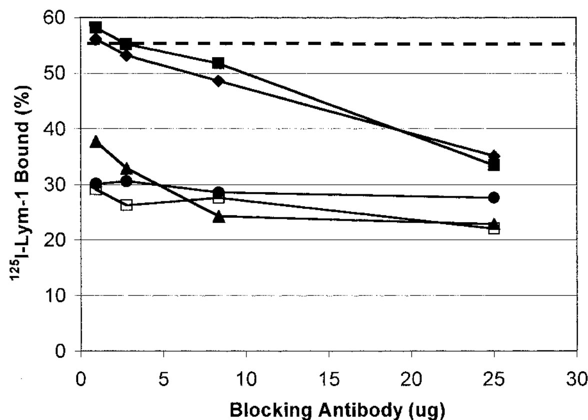

Fig. 3 Purified preparations of both Ab2 and Ab3 were capable of blocking the binding of ^{125}I-Lym-1 to Raji cells. Varying amounts of several preparations of purified immunoglobulin populations of Ab2 and Ab3 and of Lym-1 MAb were tested to determine whether they blocked the binding of ^{125}I-Lym-1 to Raji cells. ■, Ab3#4; ♦, Ab3#6; ●, Ab2#2; ▲, Ab2#6; □, Lym-1. The *dashed line* represents the level of binding of ^{125}I-Lym-1 to Raji cells in the absence of competitor.

Fig. 2. As with Ab2, different yields of Ab3 were obtained from different plasmaphoresis material. Representative activity titers of several preparations are shown in Fig. 3, in comparison with titers of Ab2 and to Lym-1 itself. Excess amounts of the Ab3 preparations were able to block the binding of Lym-1 MAb to its antigen on the surface of the Raji cell. Also, association of the Ab2 and Ab3 was shown using cellulose acetate electrophoresis. The combination of Ab2 and Ab3 migrated slower than either antibody alone (data not shown).

ADCC Assay. ADCC activity was present in all of the patient's postseroconversion plasmaphoresis samples tested, with HAMA-negative volunteer plasma as a control. The December plasmaphoresis sample gave the highest percentage of specific lysis. This result correlated with the patient's increasing levels of circulating HAMA over the course of her therapies. The Ab3 preparation, purified from the November plasmaphoresis material, was also positive for ADCC activity (Fig. 5).

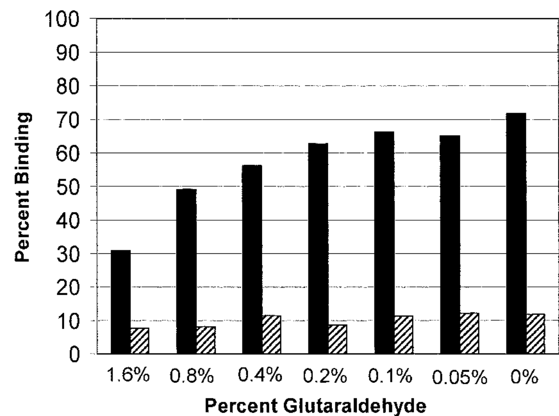

Fig. 4 Glutaraldehyde fixation of Raji cells preserved the HLA-DR10 (Lym-1 antigen) on the Raji cell surface. Raji cells were fixed with varying amounts of glutaraldehyde and then combined with ^{125}I-Lym-1 to measure Lym-1 binding to the Raji cells. Glutaraldehyde (0.5%) was determined to be the preferred fixation. *Solid bars* represent the percentage of binding of ^{125}I-Lym-1, whereas *shaded bars* represent binding in the presence of excess unlabeled Lym-1 as a competitor.

Discussion

Our data demonstrated that both Ab2 and Ab3 antibodies were induced in an NHL patient after treatments with Lym-1 MAb (Ab1). These antibodies, in purified form, possessed the activities associated with their postulated role in the idiotypic cascade. The Ab2 was capable of binding to Lym-1 and of blocking the ability of Lym-1 to bind to its antigen on the surface of Raji lymphoma cells. Although these Ab2 preparations have not been given to a xenogeneic host to induce Ab3 formation, the Ab3 purified from the same patient's plasma samples was, in theory, the resultant immunological response from within the same host. This purified Ab3 was capable of binding to the antigen on the surface of Raji cells and blocking the ability of Lym-1 to bind to this antigen. The Ab3 preparations were typically less active (on a weight per weight basis) in their ability to block this binding than were the Ab2 preparations or Ab1. This may be because of a lower association constant of the Ab3 for the antigen than that of the Ab1 or to the fact that Ab3 may express some, but not all, Ab1-related idiotopes defined by the HLA-DR10 cell surface antigen. This phenomenon was not unexpected in that the Ab3 was elicited by a mimic of the antigen and not the antigen itself.

Purified Ab3 was also capable of inducing ADCC, indicating its relevant biological activity. ADCC activity was also seen in the unprocessed plasma samples, indicating that it was present in high enough concentrations to be measured and, inferentially, to have played a role in the course of the patient's disease. Most importantly, the presence of the Ab3 human tumor-specific antibody generated by this patient's own immune system lasted for many years after treatment and was accompanied by a prolonged period of progression-free survival (14, 19). Detailed analyses and purifications were possible with this patient due to the quantities of plasma available because of her plasmaphoreses. However, the phenomenon of idiotypic cascade activation was not unique to this patient and was seen in other NHL patients treated under the same protocols in this

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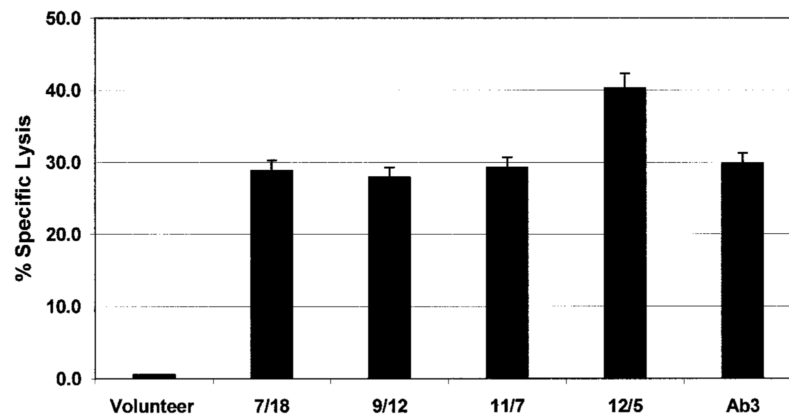

Fig. 5 Purified Ab3 from the 11/7 plasmaphoresis and the patient's plasmaphoresis material (7/18, 9/12, 11/7, and 12/5) were able to induce ADCC. Raji human lymphoma cells were used as target cells for ADCC mediated by patient Ab3 sera or purified Ab3, with normal human peripheral blood mononuclear cells as effector cells. Volunteer HAMA-negative plasma was included as a control. Error bars represent 1 SD from the mean value of triplicates.

laboratory. Patients whose HAMA titers increased during and after treatment had statistically improved survival even when adjusted for patient risk group (20). This phenomenon is consistent with the idiotypic network hypothesis in which high titers of Ab3 antibodies generated by the patient's own immune system may have a causal relationship with long-term tumor control. We have described here the first complete documentation in NHL, a cancer of the immune system, of the development of an idiotypic cascade that likely provided the patient with a survival benefit.

Groups involved in the administration of MAb for cancer therapy have postulated induction of the idiotype cascade in their patients, several with reportedly improved survival statistics (8, 21–24). Other groups have taken advantage of this postulate by directing their effort toward the application of anti-id (Ab2) vaccines for therapy of cancer (25–29). Anti-id vaccines represent an elegant and specific way to generate immunity targeted to a specific antigen. These vaccines potentially overcome the problem of a weakly-responding immune system involving immune tolerance for the tumor antigens, by presenting the antigen in an abnormal molecular format, a technique described for breaking through immune tolerance (30).

Many vaccination studies using Ab2, as cited previously, have shown inhibition of tumor growth in both animals and patients. These anti-id vaccines or internal antigen vaccines take advantage of the fact that the repertoire of external antigens is physically mimicked by id structures on immunoglobulin and possibly on receptors of T cells as well (28). Our study demonstrates that injection of Ab1 is similarly able, under favorable circumstances, to initiate the idiotypic cascade and generate biologically active Ab3 that may protect the patient for years after the Ab1 therapeutic injections.

Immunotherapy, both active and passive, is attractive as an adjunct modality in the management of cancer. However, even specific and successful immunotherapies, such as Rituxan (Genentech, Inc., South San Francisco, CA; IDEC Pharmaceutical, San Diego, CA) or Zevalin (IDEC Pharmaceutical) treatment for NHL, deplete the recipient's B cells, potentially impairing their antitumor response. A scenario for a cancer treatment strategy is that suggested by Timmerman (31), who proposed that immunosuppressive agents be deferred until later in the treatment regimen. The advantages of an immunological approach to cancer therapy are numerous. Once an immune response is triggered, prolonged self-perpetuating immunity may be achieved.

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