Development and Evaluation of the Specificity of a Rat Monoclonal Anti-Idiotypic Antibody, WN, to an Anti-B-Cell Lymphoma Monoclonal Antibody, LL2

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Abstract

Anti-idiotype monoclonal antibodies (Mabs) to mLL2, an anti-B-cell lymphoma and CD22-specific murine IgG2a-κ Mab, were generated by hybridoma technology from spleenocytes of Copenhagen rats immunized with mLL2 F(ab')2. Mab WN, an IgG2a-κ, was selected based on its specific binding to mLL2 and not other IgG isotopes or anti-B-cell Mabs. In a radioimmunoassay, WN was found to inhibit the binding of 125I-labeled mLL2 to Raji cells and to have no effect on the binding of other B-cell-reactive antibodies. Using high performance liquid chromatography analysis, WN was shown to complex specifically with both mLL2 and mLL2 Fab'. Meanwhile, we have constructed chimeric (cLL2) and humanized (hLL2) versions of LL2. Both cLL2 and hLL2 were demonstrated to retain the original antigen specificity and affinity of mLL2 [S. O. Leung et al., Proc. Am. Assoc. Cancer Res., 2872 (abstract), 34: 481, 1993]. The specific binding of WN to either radiolabeled or peroxidase-conjugated mLL2 was inhibited in a dose-response manner, and to a similar extent by mLL2, cLL2, and hLL2. Since the mLL2 complementarity-determining regions are the only sequences common to mLL2, cLL2, and hLL2, the result confirms that WN is specific to the antigen-binding complementarity-determining regions. A WN binding assay is currently being evaluated as a substitute for the tedious, and sometimes inconsistent, Raji cell-binding assay for the determination of LL2 immunoreactivity. In conclusion, we have developed an anti-idiotype Mab, WN, to mLL2. Its potential use as a surrogate antigen for B-cell lymphoma is under investigation.

Introduction

An anti-idiotype antibody (Ab2) is an immunoglobulin with specificity directed against determinants located within the variable region of an antigen-specific antibody (Ab1). Certain Ab2s, termed Ab2β, recognize an idiotope within the antigen-combining site of Ab1 and can mimic the three-dimensional structure of the antigen that generated the Ab1. In that instance, it is argued that Ab2 can, in effect, be used in lieu of that antigen.

LL2 is a murine IgG2a-κ Mab1 (1) specific for the epitope B of CD22 (2, 3) and is proven to be of clinical importance for radioimmunodetection and radioimmunotherapy of non-Hodgkin’s B-cell lymphoma (3, 4). Due to the lack of commercially available purified CD22 protein, assessment of the immunoreactivities of LL2 and its derivatives (e.g., fragments, conjugates, and chimeric and humanized LL2) have to be evaluated using Raji cells, a human Burkitt lymphoma cell line, as the source of antigen. The method is not only tedious but also inconsistent. Our aim has been to generate an Ab2β to LL2 and to use it as a supplementary tool to evaluate the immunoreactivity of LL2 and possibly as a vaccine in the treatment of lymphoma and leukemia of B-cell lineage.

The generation of syngeneic anti-idiotype Mab requires the coupling of Ab1 with keyhole limpet hemocyanin to enhance its immunogenicity (5, 6). We reported previously the generation of W12 (7), an anti-idiotype Mab to MN-14 (8), a murine IgG to CEA, using Copenhagen rats as the host and MN-14 F(ab')2 as the immunogen. In such a xenogeneic system, immunization with the intact fragment triggered a strong anti-idiotypic and anti-isotypic response limited to the constant domain of the antibody light chain (CL) and the first constant domain of the antibody heavy chain (CH1). A similar approach for the generation of an Ab2β to LL2 is described in this report.

Materials and Methods

Antibodies. The murine LL1 and LL2, raised by immunization of BALB/c mice with an extract of Raji cells, have been described previously (1, 2), and the Fab'2 fragments were prepared by pepsin digestion. A103 is an irrelevant mouse IgG2a-κ control Mab. The specificities of the other control murine Mabs, NP-4 (9), MN-3, MN-14, MN-15 (8), the chimeric LL2 (10), and humanized forms of LL2 (11) have been reported.

Immunization Protocols and Mab Production. The immunization with Ab1 was as described previously (7), except for the frequency of the injections, which were performed on days 1, 15, 21, 28, and 35. The fusion was as described (5). The hybrid supernatants were screened for the production of an IgG with positive binding to LL2 F(ab')2 and absence of binding to the mouse IgG2a-κ control Mab A103, as described below. The hybrids specific for LL2 F(ab')2 were cloned three times by limiting dilution. Ab2 was expanded in culture media and purified by affinity chromatography on a recombinant protein G-Sepharose column (Pharmacia, Piscataway, NJ).

ELISA Assays. The specificity of the purified Ab2 was determined by two ELISA assays: (a) the reactivity of the Ab2 was screened on plates coated with either LL2 or A103 IgG (50 μg/well of a 10-μg/ml solution). Affinity-purified Ab2 was diluted at 0.02 to 20 μg/ml, and 50 μl were added onto the plates. The binding of the antibody was revealed with a peroxidase-conjugated mouse antirat immunoglobulin; and (b) the specificity of Ab2 was screened by using Ab2 as the coating agent (50 μl of a 10-μg/ml solution). Mab LL2, MN-3, MN-14, MN-15, NP-3, or NP-4 (50 μl at 0.01 to 1 μg/ml) was added; and the binding of these antibodies was detected with a peroxidase-conjugated rat antirat immunoglobulin.

HPLC Analysis. The binding of Ab2 to Ab1 IgG and fragments was assessed by HPLC. 125I-labeled A103 and 125I-labeled LL2 (20 ng) were analyzed on a Zorbax GF 450 gel filtration column (DuPont, Wilmington, DE) before and after incubation with Ab2. The elution profiles of the radiolabeled agents were compared to determine the extent of the complexation, the complexes with higher molecular weights eluting earlier than the free IgG. Furthermore, LL2 Fab' was radiolabeled with 3H1, aliquots of 100 ng were incubated with Ab2 (0.4 to 32 μg), and samples were analyzed on a BioSil SEC gel filtration HPLC column (Bio-Rad, Richmond, VA).

Inhibition of the Binding of Ab1 to Raji Cells by Ab2. The ability of the anti-idiotype antibody to prevent the binding of Ab1 to Raji cells was evaluated in a radioimmunoassay. 125I-labeled LL1 and 125I-labeled LL2 (20 ng) were incubated with WN (1.95 ng to 2 μg). The mixture was added to the Raji cells (1 × 107 cells). After 15 min, the cells were washed, and bound 125I was counted.

Inhibition of Ab2 Binding to Ab2 by Murine, Chimeric, and Humanized LL2. mLL2 IgG was peroxidase conjugated by a proprietary protocol, diluted to 0.04 μg/ml, and incubated with murine, chimeric, and two humanized forms of LL2 (0.01 to 500 μg/ml). The mixture was added to ELISA
This result suggests that WN is specific to the variable domains of concentrations, WN bound solely to LL2 (Fig. 1). Furthermore, five Characterization of the Binding of WN evaluated by HPLC analysis. As shown in Fig. 3B, after incubation of ELISA assay against LL2 and the IgG2a control Mab A103. At all and was purified from culture supernatant by affinity chromatography. continued to produce an LL2-specific antibody. This clone was expanded, to the isotype-matched control antibody A103, as evaluated in the only 20 of them tested positive for specific reactivity to LL2 and not Generation of Ab2 to LL2 F(ab')2 earlier for cLL2 and hLL2. In parallel, the immunoreactivity of the conjugates was tested to evaluate if the Ab2 could be used as a surrogate antigen in the Evaluation of the Immunoreactivity of Drug-conjugated LL2 by Bind plates precoated with Ab2. The binding of the peroxidase-conjugated LL2 was determined as described (5).

Evaluation of the Immunoreactivity of Drug-conjugated LL2 by Binding on Ab2. The assay measuring the inhibition of the binding of Ab1 to Ab2 was tested to evaluate if the Ab2 could be used as a surrogate antigen in the determination of the immunoreactivity of LL2 and its derivatives. LL2 F(ab')2 was conjugated with the anticancer agent doxorubicin using the 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide condensation method (12). The conjugates were purified by gel filtration on a Bio-gel A 0.5-m column (Bio-Rad, Melville, NY). Doxorubicin conjugates with a drug substitution level of 3.7, 6.0, and 9.1 were obtained. Their immunoreactivity was evaluated as described earlier for cLL2 and hLL2. In parallel, the immunoreactivity of the conjugates was determined by the conventional Raji cell binding assay (1).

Results
Generation of Ab2 to LL2 F(ab')2
The spleen of the Copenhagen rat immunized with LL2 F(ab')2 was fused with SP2/0 myeloma cells. Of a total of 520 hybrids generated, only 20 of them tested positive for specific reactivity to LL2 and not to the isotype-matched control antibody A103, as evaluated in the ELISA assay. After cloning, only 1 clone remained stable and continued to produce an LL2-specific antibody. This clone was expanded, and the antibody designated as WN was characterized as an IgG2a-κ and was purified from culture supernatant by affinity chromatography.

Characterization of the Binding of WN
ELISA Assays. To assess its specificity, WN was tested by an ELISA assay against LL2 and the IgG2a control Mab A103. At all concentrations, WN bound solely to LL2 (Fig. 1). Furthermore, five murine IgG1 Mabs failed to show any reactivity with WN (Fig. 2). This result suggests that WN is specific to the variable domains of LL2.

HPLC Analysis. The specific binding of WN to LL2 was further evaluated by HPLC analysis. As shown in Fig. 3B, after incubation of WN with 125I-labeled LL2, the elution peak representing the LL2/WN complex appeared earlier than that of LL2 alone. However, this peak-shifting was not observed when WN was incubated with the 125I-labeled control antibody A103 (Fig. 3A). In a separate study, LL2 Fab' labeled with 99mTc was incubated with WN and subjected to radio-HPLC analysis. A similar mobility shift indicative of the formation of a WN/LL2 Fab' complex was observed (data not shown).

Competitive Binding Radioimmunoassay. Various concentrations of WN were tested for inhibiting the binding of 125I-labeled LL1 and LL2 to Raji cells. LL1, like LL2, is a B-cell-specific antibody but recognizes a different epitope (1). As shown in Fig. 4, the binding of 125I-labeled LL2 to Raji cells was inhibited by WN in a dose-response manner, whereas the binding of 125I-labeled LL1 was unaffected by WN. The result suggests that WN recognizes specific idiotypic determinants within the LL2 antigen-binding site. From the binding data on Raji cells, the affinity of WN for mLL2 was estimated to be 1 x 10^10 M^-1.

Binding to Chimeric (cLL2) and Humanized (hLL2) Antibodies. Chimeric and CDR-grafted LL2 antibodies have been constructed and described elsewhere (10, 11). The murine LL2, chimeric LL2, and the two different versions of humanized LL2 compete equally with peroxidase-conjugated LL2 for binding on WN, as seen in Fig. 5; a similar result was determined when 125I-labeled LL2 was substituted for the peroxidase-conjugated form of LL2 (data not shown). These findings suggest that Mab WN is specific to the antigen-binding CDRs, a region common to murine and genetically engineered LL2 Mabs.

Use of WN to Assay for LL2 Immunoreactivity
Different conditions to optimize doxorubicin conjugation to the F(ab')2 fragments of LL2 for immunotherapy are under investigation. The effects of the different chemical conditions on the immunoreactivity of the conjugates were assessed by comparing their binding affinities to WN. As shown in Fig. 6A, the ability of the conjugates to compete with the peroxidase-conjugated LL2 for binding on WN is
ANTI.IDIOTYPE ANTIBODY TO AN ANTI-B-CELL ANTIBODY

Discussion

The concept of "internal image," later changed to "functional mimicry," was formulated by Niels Jerne in his "network theory" of immunity in 1974 (13). Twenty years later, there are still no definitive biochemical criteria to identify the presence of an internal image determinant on an anti-idiotype antibody and to define an Ab2β. Recent studies have investigated the antigen/idiotype/anti-idiotype interaction by sequencing the variable regions of the antibodies. Some authors have attributed the antigen mimicry of Ab2 to shared structures of the Ab2 and the antigen (14–16).

markedly decreased when compared with the unmodified LL2 F(ab')2. At the three-drug substitution level (3.7, 6.0, and 9.1), the conjugates at a concentration of 0.5 μg/ml showed a dramatic loss in immunoreactivity, and at a concentration of 1 to 5 μg/ml, the drop was inversely related with the level of drug substitution. For comparison, the same conjugates and LL2 F(ab')2 at various concentrations were tested for their binding on Raji cells. Cell surface-bound antibodies or conjugates were detected with a FITC-labeled goat antimouse antibody in a flow cytometry assay. As shown in Fig. 6B, a trend similar to that shown in Fig. 6A of the effect of drug conjugation on the immunoreactivities of the resultant conjugate was observed. It should be noted, however, that the two experiments were performed differently (direct binding on WN versus indirect detection through a second antibody) and with different concentrations (10-fold difference). Therefore, the absolute value for the percentage decrease in immunoreactivity corresponding to the number of drugs conjugated should not be compared.

Fig. 3. Radiolabeled Mabs A103 (A) and LL2 (B), 20 ng, were incubated with WN, 2000 ng, for 2 h at room temperature. Mab and Mab/WN were analyzed by HPLC with a Zorbax GF 450 gel filtration column. Fractions were collected, and 125I was counted.

Fig. 4. Mabs to Raji cells. LL1 and LL2, were radiolabeled with 125I. 125I-labeled LL1 and 125I-labeled LL2, 20 ng, were incubated with WN, 1.95 ng to 2 μg. Raji cells were prepared from suspension cultures; then 125I-LL1/WN and 125I-LL2/WN were added to the cells (1 × 10⁶) and incubated for 15 min at room temperature. The cells were washed, and the bound 125I was counted.

Fig. 5. ELISA plates were coated with WN, 10 μg/ml. Peroxidase-conjugated mLL2 (0.04 μg/ml) was mixed with mLL2, cLL2, hLL2-1, hLL2-2 purified from culture media, hLL2-2 (BioR) prepared in a stirred tank bioreactor, or NP-4 as control, 0.01 to 50 μg/ml. The mixture was added to the plates. After 1 h, the binding of peroxidase-conjugated mLL2 was measured.

Fig. 6. Cell surface-bound antibodies or conjugates were detected with a FITC-labeled goat antimouse antibody in a flow cytometry assay. As shown in Fig. 6B, a trend similar to that shown in Fig. 6A of the effect of drug conjugation on the immunoreactivities of the resultant conjugate was observed. It should be noted, however, that the two experiments were performed differently (direct binding on WN versus indirect detection through a second antibody) and with different concentrations (10-fold difference). Therefore, the absolute value for the percentage decrease in immunoreactivity corresponding to the number of drugs conjugated should not be compared.
Conversely, Leu et al. (17) have reported an Ab2 mimicking the anti-cancer drug taxol in its physiological properties, although no structural similarities between the Ab2 and taxol exist. In our laboratory, we have previously generated W12, an Ab2 to an anti-CEA Mab which could generate in mice and rabbits an anti-CEA response (7), and a chimeric W12. As in the study by Leu and associates, no obvious similarities between the CDRs of W12 and CEA have been found. The report of Bentley et al. (18) is in agreement with the findings of Leu et al. (17) and of Leung; using X-ray diffraction methods, the authors studied the comparative structure of an Ab1/antigen complex and an Ab1/Ab2 complex and found that the surfaces of the antigen and Ab2 do not resemble each other, although they both interact with the same residues in the binding site of Ab1. Thus, to date, structural analysis cannot predict the functional mimicry properties of an Ab2, and the immunological characteristics are the only criteria to identify if an Ab2 is truly an Ab2.

We described here the generation of WN, a rat Mab against the CD22-specific LL2 antibody, that functions immunologically as an Ab2. Characterization of the antibody has shown the following: (a) WN is exquisitely specific for LL2 and reacts with no other Mab (Figs. 1 and 2); (b) WN is capable of inhibiting the binding of LL2 to Raji cells and the inhibition is specific, since the binding of LL1 (1), another B-cell-specific antibody to Raji cells, was not affected by WN (Fig. 4); (c) the HPLC experiments prove that WN binds to LL2 by its Fab′ fragment (Fig. 3); (d) similar binding on WN have been found. The report of Bentley et al. (18) is in agreement with the findings of Leu et al. (17) and of Leung; using X-ray diffraction methods, the authors studied the comparative structure of an Ab1/antigen complex and an Ab1/Ab2 complex and found that the surfaces of the antigen and Ab2 do not resemble each other, although they both interact with the same residues in the binding site of Ab1. Thus, to date, structural analysis cannot predict the functional mimicry properties of an Ab2, and the immunological characteristics are the only criteria to identify if an Ab2 is truly an Ab2.

Chimeric LL2 is comprised of the murine LL2 VH and VL domains joined to the human IgG1 and κ constant regions (10). The competitive binding to WN of murine and chimeric LL2 confirms that the specificity of WN is not to the constant but to the variable regions of LL2. The humanized LL2 Mabs (11) were constructed by grafting the CDRLs of LL2 onto the human antibodies EVH and REI VL frameworks. Since the only common sequences shared by the murine, chimeric, and humanized LL2 Mabs are their CDR sequences, their comparable binding patterns to WN argue that WN binds specifically to the antigen-binding site, or the idiotope, of LL2. This finding supports that WN is an Ab2β antibody of LL2.

The availability of an Ab2β for LL2 has facilitated our efforts in developing LL2 as a diagnostic and therapeutic agent, which was hindered by the lack of a reliable simple and reproducible in vitro immunosassay method. The use of WN as a surrogate antigen in HPLC or ELISA assays has drastically increased the sample size tested, reduced the experimental time, simplified the procedure, and improved the sensitivity. As reported (Fig. 6A), the ELISA assay indicated a loss of immunoreactivity of doxorubicin-LL2, which was not as obvious using the Raji cell-binding assay (Fig. 6B). We are presently using the WN ELISA assay for assessing the chemical conditions for drug or radionuclide conjugation to LL2. Although we show that the specificity of WN is directed against the antigen-binding site of LL2, we do not have definitive proof that the antibody mimics the CD22 B-epitope. Although preliminary data are suggesting the production of anti-sera specific for Raji cells in BALB/c mice immunized with WN, we appreciate that WN should be used only in the preliminary screening of the immunoreactivity of LL2, and final confirmation with the standard Raji cell-binding assay is necessary. Accordingly, further studies on WN as the surrogate antigen for the antigen epitope of LL2 and as a therapeutic vaccine for B-cell lymphoma are under way.

References

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