Antibodies to Shared Idiotypes as Agents for Analysis and Therapy for Human B Cell Tumors


Monoclonal anti-idiotypic antibodies generated against idiotypic immunoglobulin (Ig) of neoplastic B lymphocytes can be selected from growing hybridoma clones by their ability to recognize idiotypic but not normal IgM. This group of antibodies can be subdivided into those that bind to the target tumor cells in the presence of normal human serum (~85% of the clones) and those in which binding is inhibited by serum (~15%). The former appear to be specific for private idiotypic determinants whereas the latter recognize cross-reacting idiotypic determinants. Such cross-reactivity is reflected both in recognition of a small percentage of normal Ig and also in binding to other lymphomas. The anti-idiotypes specific for private determinants can be used for therapy, with only idiotyp Ig secreted by tumor cells able to block its access to cells. The cross-reacting anti-idiotypes will face in addition the barrier of the proportion of normal Ig with which it reacts. The attraction of using a single monoclonal reagent for more than one patient has led us to develop an assay that measures the level of such blocking and to propose that those recognizing <30 μg/mL of normal Ig could be placed in a panel for possible therapy for several patients; less restriction need apply to antibodies for monitoring tumor progress.

The assay is described, and examples of such antibodies raised against lymphoma cells from two patients are given together with comparisons with them of anti-idiotypes specific for private determinants.

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THE POSSIBILITY of using anti-idiotypic antibodies in the therapy of human B cell lymphoma has been investigated, and there has been one apparent success. However, there are problems remaining, such as the ability of the tumor cells to endocytose surface idiotypic antibody and thereby escape from antibody attack. There is also the problem of the presence of small amounts of secreted idiotyp in the plasma that can block antibody and prevent attachment to the cells. A recent clinical trial of anti-idiotypic antibody therapy has been reported and demonstrates the limits of the approach using simple bivalent mouse monoclonal antibody. The use of univalent antibody consisting of Fab' γ exmonoclonal antibody linked chemically to host IgG allows binding to target cells but prevents endocytosis. Such a molecule can invoke both complement-mediated lysis and antibody-dependent cellular cytotoxicity and has reduced immunogenicity compared with whole mouse antibody. Preliminary tests in vivo have shown good survival of the molecule with a half-life dictated by the attached host IgG, and therapeutic trials are in progress.

However, even if this modified approach is successful, it will still be necessary to raise individual monoclonal anti-idiotypic for each patient since the chance of two separate tumors having the same set of idiotypic determinants is extremely low if the incidence of such tumors reflects the full B cell repertoire. Idiotypic determinants cover a wide range of antigenicity from the private to the semipublic, and presumably the complementarity-determining regions (CDRs) involved in binding to antigen would give rise to the most private determinants. It might be expected that antibodies of similar specificities would show similar idiotypic determinants that cross-react, and this has been shown, e.g., for rheumatoid factors and for cold agglutinins. However, other cross-reactions might occur outside the antigen-binding site because of the presence of less private determinants, and this suggests the tempting possibility of using such an antibody to monitor and perhaps treat more than one patient. However, this approach encounters the problem that a determinant semipublic as regards its occurrence on lymphocytic surfaces is likely also to be semipublic in its occurrence on extracellular Ig. Its presence there would presumably block access of antibody to target cells in the same way as tumor-derived idiotypic Ig is able to do, and therefore an additional barrier would be present. This report describes an assay that will give some measure of the degree of cross-reactivity of such antibodies and includes an estimate of the levels of blocking anticipated. If an arbitrary limit of, e.g., <30 μg/mL of blocking Ig is imposed, it might be possible to create a panel of anti-idiotypic that can be monitored on frozen sections of lymph nodes of a wide range of patients with a view to use as monitoring and perhaps therapeutic agents. This report is concerned with analysis of typical monoclonal anti-idiotypes reacting with both private and cross-reacting determinants against the lymphoma cells of two patients.

MATERIALS AND METHODS

Patients and cell preparations. Two male patients, S.A. and N.L., aged 66 and 56 years respectively, both presented with follicular lymphoma of low-grade histology. S.A. had fairly widespread tumor present in lymph nodes and a WBC count of 7 x 10^9/L, approximately 50% of which were neoplastic B cells. N.L. had skin and spleen involvement and a WBC count of 10 x 10^9/L consisting of 90% neoplastic B cells. Tumor cells were obtained from an excised inguinal lymph node from S.A. and from the blood of N.L. by described procedures, and examination of surface Ig showed that cells from both patients expressed IgM/α with a trace of IgD.
Preparation of idiotic IgM. Idiotypic IgM as a source of antigen for raising anti-idiotypic antibody was rescued from tumor cells by fusion with the mouse myeloma line NS-1 (P3-NS-1/1 Ag 4.1) (patient S.A.) or 653 (P3X63 Ag 8-635) (patient N.L.). After fusion, cells were distributed into a 96-well microculture plate at \(-2 \times 10^3\) per well on a feeder layer of irradiated mouse thymocytes. Colonies producing human IgM were cloned at regular intervals in HT medium (HT - 10\(^{-7}\) mol/L hypoxanthine, 1.6 \times 10^{-3}\) mol/L thymidine) by limiting dilution to guard against spontaneous loss of Ig chain production. Analysis of the rescued IgM on Ultragel ACA 22 showed it to be predominantly in the pentameric form.

Monoclonal anti-idiotypes. For each patient, spent xenohybridoma culture supernatant containing rescued IgM was used as a source of immunogen for immunization of Balb/c mice (N.L.) or Lou rats (S.A.). Idiotypic IgM was prepared from supernatants as purified immune complexes with mouse or rat antimouse Fab\(\alpha\) serum by the method described for production of sheep polyclonal anti-idiotypic. Each animal was immunized with complexes containing 10 to 50 \(\mu\)g of idiotypic IgM in 0.2 mL CFA distributed among four subcutaneous sites. A booster intravenous (IV) injection of antigen in 0.2 mL aqueous solution was given 2 weeks later and immune spleen cells prepared three to four days after this. These were fused with NS-1 or 653 as for the rescue of IgM, and antibody-producing cloned hybridoma cells were expanded in spinner flasks.

Idiotypic IgM: Enzyme-linked immunosorbent assay (ELISA). This technique was used to monitor production of rescued human IgM from the xenohybrids and was carried out as described. The coating antibody was rabbit antihuman \(\mu\)-chain (10 \(\mu\)g/mL), and detection was with horseradish peroxidase (HRP)-conjugated rabbit antihuman \(\lambda\)-chain (Dako, Copenhagen). A purified IgM macroglobulin was used as a standard.

For assessment of monoclonal anti-idiotypes a capture assay was used in which plates coated with rabbit antihuman \(\mu\)-chain were treated with culture supernatant containing rescued IgM or normal IgM at 100 ng/mL. Undiluted anti-idiotypic-containing culture supernatant was then loaded before detecting with HRP-rabbit antihuman IgG (1:1000) (Nordic Labs, Ltd, Maidenhead, Berkshire, UK) or HRP-sheep antitrat Ig, as appropriate. Antibodies that showed strong reactivity with idiotypic IgM but no reactivity with normal IgM were selected for a second assessment by immunofluorescence on target cells. If binding to cells was inhibited by normal human serum, the antibodies were suspected to be against cross-reacting idiotypes, and further assessment by ELISA using panels of monoclonal IgMs and IgGs as antigens was carried out. An inhibition assay was established to quantitate the degree of cross-reactivity of the various anti-idiotypes with normal IgM or IgG. For this, idiotypic IgM was coated directly on a microtiter plate, and the mouse monoclonal anti-idiotypic was mixed with varying amounts of homologous idiotypic IgM, cross-reacting idiotypic IgM, or normal IgM or IgG. The amount of mouse antibody able to bind to the coated antigen under these conditions was then measured by addition of HRP-rabbit antitrat Ig.

Preparation of IgM paraprotein from serum. The IgM paraproteins found to cross-react with anti-idiotypes were purified from patients' sera for use in the direct ELISA by chromatography on a column of Ultragel AcA 22 (LKB, Bromma, Sweden). The two patients, Ha and Ru, each had a clinical picture of Waldenström's macroglobulinemia with an IgM serum paraprotein. Patient Ha had an IgM paraprotein at 53 g/L with cold agglutinin activity of anti-I type, and patient Ru had an IgMA paraprotein at 16 g/L with no detectable antiautomunobody activity. In each case, since the paraprotein was present at high levels in the serum, separation on Ultragel AcA 22 was sufficient to yield purified pentameric IgM. Normal IgM used for screening hybridoma supernatants was prepared from pooled human serum by immunosorption as described previously.

Immunochemicals. Testing of the anti-idiotypes on the patient's dispersed tumor cells was carried out by the FACS III (Becton Dickinson Electronics, Mountain View, Calif). For inhibition studies, normal human serum (50 \(\mu\)L) was added to culture supernatants (50 \(\mu\)L) containing anti-idiotypic at \(-20 \mu\)g/mL before adding to the cells. Bound mouse or rat antibody was detected with fluorescent rabbit antimouse Ig or sheep antirat Ig as appropriate.

Tissue sections. Frozen tissue sections were used to assess reactivity of the anti-idiotypic antibodies with tonsil, reactive lymph node, and lymph nodes from patients with lymphoma. Cryostat sections (5 \(\mu\)m) were air dried at room temperature and stored at \(-70^\circ\)C until required. Immediately before staining, the sections were fixed in acetone for ten minutes and then stained by a two-layer immunoperoxidase technique using HRP-rabbit antimouse Ig or HRP-sheep antitrat Ig as the second layer and developing reaction product with diaminobenzidine tetrahydrochloride.

Cold agglutinins and hemagglutination inhibition. Other IgM paraproteins with or without cold agglutinin activity have been described previously except proteins Cho, Dav, Ham, and Cab, which are noncold agglutinin proteins from patients with Waldenström's macroglobulinemia. For the indirect hemagglutination assay, the cold agglutinin Den, which is an IgMA with anti-i activity, was coated to sheep RBC using tannic acid.

The anti-idiotypic antibody was then found to agglutinate coated cells, and using a convenient dilution of antibody (\(-0.3 \mu\)g/mL), the titers of proteins giving inhibition of agglutination were measured.

RESULTS

Preliminary screen for anti-idiotypes. Culture supernatants from growing clones were assessed by ELISA for anti-idiotypic activity, and those showing strong reactivity with the patient's idiotypic IgM and no reactivity against normal IgM were selected for expansion. Typically, out of five plates usually tested, supernatants from about 20% of the wells fulfilled this criterion of specificity.

The second part of the preliminary screen is to test for binding to target tumor cells by immunofluorescence in the presence of or absence of normal human serum (NHS). The majority (\(\geq 90\%\)) of supernatants reacting with idiotypic IgM in the ELISA also showed binding to target tumor cells; residual negative supernatants were not followed up, but apparent lack of binding could have been due to a low concentration. The results obtained for anti-idiotypic against patient S.A. are shown in Figs 1 and 2. In Fig 1 the degree of fluorescence obtained with anti-Id (1) is seen to be unaffected by the presence of NHS or S.A. serum (2 and 3), indicating recognition of private determinants, whereas in Fig 2, the similar fluorescence obtained with anti-cross-reacting id (anti-IdX) (1) is completely abrogated by NHS or S.A. serum (2 and 3). The conclusion therefore is that the anti-IdX recognizes a minor component of normal Ig insufficient to be recognized in the ELISA, but sufficient in 50% NHS to inhibit binding to cells. The incidence of such antibodies generated is variable, with an average from seven successful fusions for anti-idiotypic production of 10% to 15% of the selected anti-idiotypes. One anti-Id and one anti-IdX were then studied for each patient.

Secondary screen for anti-IdX. Using the capture assay and a random series of sera from patients with macroglobuli-
Fig 1. Binding of anti-idiotypic antibody raised against S.A. (anti-Id S.A.) to target lymphoma cells from S.A. and the effect of serum on this. Tumor cells were exposed to anti-idiotyp (20 μg/mL) mixed with an equal volume of (1) phosphate buffered saline (PBS), (2) normal human serum, or (3) serum from S.A. Bound antibody was detected with fluorescent rabbit antirat IgG (0.5 mg/mL). (4) Control of normal rat IgG.

Fig 2. Binding of cross-reacting anti-idiotypic antibody raised against patient S.A. (anti-IdX S.A.) to target lymphoma cells from S.A. and the effect of various agents on this. Tumor cells were exposed to anti-idiotyp (20 μg/mL) mixed with an equal volume of (1) PBS, (2) normal human serum, (3) serum from S.A., and (4) idiotypic IgMx (1 mg/mL) from a patient with macroglobulinemia and cold agglutinin disease.
nemia or myeloma chosen for high levels of paraprotein and assayed at the same Ig concentration as homologous idiotypic Ig (100 ng/mL), the results shown in Table 1 were obtained. In this, the antibodies against anti-IdXs, defined by blocking of reactivity with homologous lymphoma cells in the presence of normal serum, show a limited degree of reactivity in the panel. The anti-IdX from patient S.A. recognized one of 13 IgM paraproteins, and this was an IgMx with cold agglutinin activity. Since the idiotypic IgM from S.A. was IgMλ, this indicates no involvement of light-chain type in recognition. For the anti-IdX from patient N.L., three of 42 IgM paraproteins were positive, and these were one IgMx and two IgMλs, again demonstrating that cross-reactivity is independent of light-chain type. The IgMx recognized was different from that which reacted with the antibody against patient S.A. The anti-IdX from N.L. also reacted with an IgG paraprotein, demonstrating independence of the heavy-chain class also. Under the conditions of the assay, reactivities were strongly positive (E_{50} > 1.0) or negative, with no intermediate values.

Since anti-IdX S.A. recognized an anti-I cold agglutinin, a further investigation was made into reactivity with a panel of cold agglutinins directed against I or i antigens (Table 2). As shown, the antibody reacted with seven out of eight of the cold agglutinins, with the negative result being for IgM Ma, a protein known to lack the majority of idiotypic determinants shared by this family of cold agglutinins. One of the proteins lacking cold agglutinin activity, but known to share idiotypic determinants with anti-I and anti-i cold agglutinins, IgM Mar, also bound to the antibody. The idiotypic IgMλ rescued from the lymphoma cells of S.A. showed no cold agglutinin activity.

To localize the cross-reacting idiotypic determinants by a different procedure, the binding of anti-IdX to cells from patient S.A. was assessed in the presence or absence of the IgMx cold agglutinin (Ha) or its Fabα derivative prepared by papain digestion. Both the whole IgM and the Fabα gave total inhibition of fluorescence when at 0.25 mg/mL, whereas they had no effect on binding by the non-cross-reacting anti-Id.

**Quantitation of cross-reactivity.** The inhibition ELISA used to measure the proportion of normal Ig recognized by anti-IdX S.A. is shown in Fig. 3. For the assay, since the IgMx macroglobulin recognized by the antibody reacted at the same level as the rescued IgMλ S.A., the former was coated onto the plate, and it can be seen that both this IgMx and the rescued idiotypic IgMλ from S.A. are equally efficient at inhibiting the binding of anti-idiotype to the IgMx on the plate, with ~4.5 ng/mL giving 50% inhibition (Table 2). However, normal IgM and IgG are considerably less efficient, and assuming equal efficacy of monomeric IgG and polymeric IgM in the assay, approximately one in 200 molecules of IgG and one in 600 molecules of IgM carry the cross-reacting idiotype. In a normal serum this would represent 60 μg/mL of IgG and 2 μg/mL of IgM. The fact that the curves for specific idiotype, cross-reacting idiotype, and

| Table 1. Reactivities of Monoclonal Anti-Idiotypes With Human Paraproteins |
|-----------------------------|---------|---------|
| Anti-idotype                  | IgM     | IgG     |
| Anti-id S.A.*                | 0/13    | 0/26    |
| Anti-id S.A. †               | 1/13    | 0/26    |
| Anti-id N.L.*                | 0/42    | 0/26    |
| Anti-id N.L. †               | 3/42    | 1/26    |

*Antibody against private idiotype.
†Antibody against cross-reacting idiotype.

The IgM paraprotein recognized by anti-IdX S.A. was not recognized by anti-IdX N.L.
normal IgG and IgM are parallel (Fig 3) strongly suggests that similar antigenic determinants are being recognized on all the competing Igs. There is no suggestion of interference by heterophile antibody in human serum that might recognize mouse Ig, and even if this were present, it should not interfere with the binding of anti-idiotypic to idiotype.

Since the tumor cells from S.A. secrete no detectable Ig, it was possible to compare the abilities of normal serum from patient S.A. and pooled normal human serum to inhibit the aforementioned reaction. They were found to be equally effective, which suggests that the antibody is not allotypic. This is also suggested by the heavy- and light-chain independence in the patterns of reactivity, leaving cross-reacting antigen in the variable region. Allotypic determinants in this region cannot easily be distinguished from framework determinants or less private idiotypic determinants.\textsuperscript{16}

Comparable data, including the anti-allotype control, was obtained for the anti-IdX N.L., and the amounts required for the 50% inhibition are also shown in Table 3. The reason for the differences in concentrations of idiotypic IgMs required for 50% inhibition (4.5 and 460 ng/mL for S.A. and N.L. respectively) probably reflects the variable efficiency of the coating of the two IgM preparations to the plastic plate. This, however, does not affect the comparison of homologous and cross-reacting idiotypes in the competitive assays. In the case of N.L., the serum would represent a barrier of 23 μg/mL of IgG and 2 μg/mL of IgM.

**Table 3. Quantitation of Cross-reacting Idiotypic Ig by an Inhibition ELISA**

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgM Id</th>
<th>IgM IdX</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.A.</td>
<td>4.5</td>
<td>4.5</td>
<td>2.8 x 10^2</td>
<td>7.5 x 10^2</td>
</tr>
<tr>
<td>N.L.</td>
<td>460</td>
<td>460</td>
<td>2.3 x 10^8</td>
<td>2.0 x 10^8</td>
</tr>
</tbody>
</table>

*Cross-reacting IgM idiotype.
†Normal IgM.

The ELISA depends on cross-reacting idiotypic molecules in the normal Ig populations binding to the monoclonal anti-idiotypic, thereby preventing its uptake by idiotypic Ig bound to the plate. The concentrations giving 50% inhibition are calculated from the inhibition curves (see Fig 3).

A similar analysis was carried out with anti-idiotypic antibodies to patient N.L. In this case neither anti-Id nor anti-IdX reacted with tumor cells in any of the 12 biopsy specimens from patients with lymphoma, although a few of the residual normal cells reacted as expected. The anti-idiotypes also did not react with the biopsy specimen from patient S.A.

**DISCUSSION**

Since the description of attempted therapy of human B cell lymphoma with anti-idiotypic antibody\textsuperscript{1} and particularly
since the reported apparent success in one patient, a number of groups have raised such antibodies for treatment. However, as well as problems of mechanisms of tumor cell escape from antibody attack, it has been shown that the presence of idiotypic IgM in the plasma, arising from low levels of secretion by the neoplastic cells, is a disadvantage in the application of this therapy. Anti-idiotype must traverse the serum to reach the tumor cells, and if it is consumed by idiotype, more antibody is required, and the immune complexes generated can consume effector mechanisms and perhaps cause toxic reactions.

Theoretically the situation is worse if the antibody recognizes more public idiotypes since they will correspondingly be present in the normal immunoglobulin, and it can be envisaged that antibodies to increasingly public idiotypes will recognize more lymphomas but also more of the serum Ig. In view of this, the screening method in this laboratory examines reactivity of antibody with target cells in the presence of normal serum. Clearly the screening procedure must be set in an appropriate way to select the antibody specificities desired, and this is the reason for the two-stage assessment using first, either idiotypic IgM or normal IgM as assessed by ELISA, followed second by the test on target cells in the presence of serum. The cross-reacting anti-Ids pass the first test but fail the second. It is difficult to assess why other groups produce different proportions of such specificities, and sometimes no cross-reacting anti-Ids but differences in the nature of the immunogen and in immunization and screening methods must be considered. Immunization with soluble idiotypic IgM rather than cells should give a larger range of antibodies that can be surveyed for such activity; this is probably necessary since the incidence in this study was 10% to 15% of putative anti-idiotypes. Some investigators have used screening against a variety of lymphomas and normal cells by immunofluorescence, which is not rigorous, and antibodies to cross-reacting idiotypes have been selected and proposed for therapy with no measure of the serum barrier that exists.

However, the attraction of using a single anti-idiotype for more than one patient remains and has led us to establish a test for the degree of cross-reactivity shown by the numerous antibodies (10% to 15% of selected anti-idiotypes) that fail our test in the presence of serum. Two typical examples have been analyzed, and the extra barrier that must be overcome for these antibodies to traverse the serum has been quantitated. If such measurements are made, it might be possible to create a panel of the more specific end of the spectrum, eg, those antibodies that recognize an arbitrary value of <30 μg/mL of normal IgG, with a view to therapy and, certainly, to use them for monitoring tumor cell spread. Such a barrier should not create major problems for antibody infusion, and it could be lowered further by plasmapheresis. For application of the test in which rescued idiotypic Ig has not been prepared, Fabʹy prepared from the surface Ig of intact cells or a tumor cell lysate could be used. Since such antibodies probably recognize framework determinants in the variable region rather than those involved in putative antigen-binding (CDRs), it might be of interest to see whether the expression of such determinants is affected by mutational events at the same frequency as appears to occur for more private idiotypes.

A further bonus of investigating these antibodies is the finding of unexpected activities such as that shown by the anti–cross-reacting idiotype produced against idiotypic IgM from patient S.A. that appears to recognize cold agglutinins. The pattern of reactivity seen is very similar to that of a monoclonal antibody specifically raised against IgM Mar known to express such cross-reacting idiotype determinants. Such reagents might provide useful insights into the range of idiotypic determinants found in lymphoma and autoimmune diseases in relation to those in the normal immunoglobulin pool.

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