Idiotype as a Tumor-Specific Marker in Childhood B Cell Acute Lymphoblastic Leukemia

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Immunoglobulin (Ig) or idiotype (Id) is a tumor-specific target in those B cell malignancies that express this molecule on their surface. We explored the biology of B cell acute lymphoblastic leukemia (B cell ALL) using Id as a tumor marker. In this report we describe the development of anti-Id monoclonal antibodies (MAB) for two children with B cell ALL. These reagents were used retrospectively to study tumor kinetics and to detect residual disease after chemotherapy. In both cases serum Id values were strikingly high at diagnosis (1.2 mg/mL and 10.8 mg/mL), suggesting that the tumor cells were relatively mature B cells capable of significant antibody production. In both patients the serum Id levels fell with the institution of therapy and confirmed that the patients were in remission. Increasing serum Id predicted relapse four months before conventional methods in patient 1, and Id proved to be a more sensitive measure of tumor burden than Southern blot analysis of rearranged Ig genes in bone marrow samples. Surprisingly, low levels of Id were redetected in the second patient just before completing therapy and have persisted for over a year despite the absence of clinical evidence of recurrent disease. Thus, serum Id levels reflect tumor burden during initial therapy but may not necessarily predict tumor progression after a complete clinical remission.

MATERIALS AND METHODS

Case 1

The patient was a 13-year-old white girl who presented to Stanford University Medical Center with a 4-week history of jaw pain and a two-week history of easy bruising and epistaxis. Physical examination revealed petechiae, mandibular swelling, and moderate hepatosplenomegaly. Her hemoglobin (Hb) was 7.9 g/dL, platelets 19,000/µL, and WBC count 13,800/µL with 24% blasts. The bone marrow was replaced by a uniform population of lymphoblasts with typical L1 FAB morphology. Immunologic analysis distinguished these cells to have surface Ig with µ phenotype. Serum LDH was 1,145 IU/L, and the uric acid was 12 mg/dL. Serum immunoglobulin levels (mg/dL) were IgG:800; IgA:80; and IgM:165. She was started on a multiagent chemotherapy regimen, including intense CNS prophylaxis. A complete remission was documented on day 28 from diagnosis. However, an isolated CNS relapse developed nine weeks from diagnosis, which was successfully treated with further intrathecal chemotherapy. Because of her extremely poor prognosis it was elected to perform autologous bone marrow transplantation (BMT). Eight months from her diagnosis remission bone marrow was harvested and treated with anti-B, MAB. The patient received high-dose cyclophosphamide and fractionated total body irradiation (TBI) followed by marrow reinfusion. One month later peripheral blasts appeared, and a bone marrow showed complete replacement with L1 lymphoblasts. Two weeks later she expired from CNS hemorrhage.

Case 2

A 7-year-old boy presented with a three-week history of generalized malaise, intermittent fever, and night sweats. Physical examination revealed bilateral periorbital swelling and hepatosplenomegaly. His Hb was 9.0 g/dL, WBC count 6,600/µL (with 5% blasts), platelets 101,000/µL. The uric acid was 29 mg/dL with an LDH of 681 IU/L. Bone marrow aspiration showed complete replacement with L1 lymphoblasts, and these cells were shown to express surface Ig (µ). He was also started on the same intensive chemotherapy regimen as patient 1. Serious metabolic problems resulting from...
tumor lysis occurred, requiring mechanical ventilation and peritoneal dialysis. A complete remission was documented on day 28, and he received 18 months of continuation treatment without further complication. Since completing therapy 12 months ago he has been in good health, with normal blood counts and no clinical evidence of recurrent leukemia.

**Cell lines.** Malignant B cells from both patients were cultured, and cell lines were established by a modification of methods described previously. Briefly, leukemic cells from bone marrow obtained at diagnosis were separated by Ficoll-Hypaque (FH) gradient centrifugation and suspended in media with agar over a feeder layer containing 10% normal human serum. Cells were eventually weaned from a feeder layer and were adapted to growth in suspension culture with RPMI 1640 (JR Scientific, Woodland, CA), 15% fetal calf serum (FCS), 50 μg/mL penicillin, 100 μg/mL streptomycin, and 2 mmol L glutamine (complete media) in a 6% CO₂ incubator.

**Recovery of tumor idiotype.** At diagnosis bone marrow was harvested, and mononuclear cells were recovered by FH density centrifugation. Cells were washed, resuspended in 10% dimethyl sulfoxide (DMSO) 90% FCS and frozen in liquid nitrogen prior to use. Leukemic cells were thawed and washed prior to fusion.

To recover Id protein, leukemic cells were fused with a nonsecreting mouse-human hybrid myeloma line, K6H6/B5, by methods described previously. The resulting hybrids were screened for the production of immunoglobulin chains μ and λ (the patient’s original phenotype) by enzyme-linked immunosorbent assay (ELISA). Positive wells were cloned by limiting dilution using irradiated Balb/c mouse spleen cells as a feeder layer.

Id was purified from the supernatant of Id-secreting clones by affinity chromatography using a goat antihuman μ reagent (Tago, Burlingame, CA) coupled to sepharose beads (Pharmacia, Piscataway, NJ). In the first case Id was purified from a single Id secreting hybrid, whereas in case 2 supernatant from several Id-secreting hybrids were pooled before harvesting Id.

**Anti-idiotype antibodies.** Purified Id was used as an immunogen to generate anti-Id antibodies. Balb/c mice were injected intraperitoneally (IP) with 50 μg Id in mixed complete Freund’s adjuvant followed by another intravenous (IV) dose of 50 μg in phosphate-buffered saline (PBS) seven days later and a final dose of 50 μg in PBS IV on day 14. Spleens were harvested on day 17 and were fused to 25 X 10⁵ Ag 8.653, a nonsecreting mouse myeloma [HRP]; Tago). To exclude MAB specific for constant-region determinants, supernatants positive by the preceding assay were then tested against Id from four unrelated B cell tumors. Antibodies that were negative in this assay would presumably be directed against idiotypic determinants, and these were then tested for whole-cell binding on the tumor cell line by indirect immunofluorescence. An affinity purified goat antiguinea Id reagent coupled to fluorescein isothiocyanate (Tago) was used as a second step. Finally, specificity was further assured by screening for nonreactivity on fixed tissue sections of normal human tonsil by the immunoperoxidase technique. Eventually anti-Id antibodies were used to stain leukemic cells harvested at diagnosis or relapse.

**Serum idiotype assay.** Anti-Id antibodies were used to screen serum samples retrospectively for Id. Anti-idiotype antibodies purified from mouse ascites by ammonium sulphate precipitation were diluted to a concentration of 10 μg/mL in PBS and were used to coat plastic microtiter plates (Immulon TM2, Dynatech Laboratories, Alexandria, VA) at 4°C overnight. Plates were washed with 0.15 mol/L NaCl, 0.05% Trition X 100, and were blocked with PBS 2% nonfat milk at room temperature (RT) for one hour. Plates were washed and serum was added in serial dilutions and was compared to a standard of affinity purified Id diluted in normal human serum or PBS 2% bovine serum albumin (BSA). A third step of goat antihuman λ HRP (light chain phenotype of both patients; Tago) was used to detect bound Id. At least one sample per plate was also tested using a third step consisting of goat antihuman μ HRP to determine nonspecific background binding of human Ig. For case 2 comparison assays were also done using goat antihuman μ HRP (Tago) to distinguish bound Id to ensure that a monoclonal protein was being detected. After a one-hour incubation at 20°C, plates were washed, and enzyme substrate (2,2 azino-di [3 ethylbenzthiazoline sulfonic acid] 150 mg/mL; Sigma) was added. Absorbance was measured on an ELISA spectrophotometer at a wavelength of 405 λ (automated micro ELISA reader; Dynatech Laboratories). Each sample was tested in two to four independent assays. This assay will detect Id levels down to 0.1 to 0.5 μg/mL.

**Southern blot analysis of rearranged Ig genes.** DNA was extracted from bone marrow mononuclear cells that had been isolated by FH centrifugation. Three samples from case 1 were analyzed: one harvested at diagnosis and two samples taken at separate time points when Id was first detected in the serum and there was no other evidence of systemic relapse. In addition, DNA was also prepared from the Id-producing hybrid used in making the anti-Id antibody. Ten micrograms of DNA was digested with restriction endonuclease Eco RI (New England Biolabs, Beverly, MA) overnight at 37°C, and samples were then electrophoresed on a 0.8% agarose gel. DNA was transferred to a nitrocellulose filter (Genatran 45, Plasco, Inc, Woburn, MA) and probed with a 32P-labeled 6.5-kb genomic sequence homologous to the J₄ heavy chain of the Ig heavy chain by methods described previously.

**RESULTS**

**Anti-Idiotype Antibodies**

For both cases multiple Id-producing hybrids were isolated from fusions between tumor cells and the heterohybridoma K6H6/B5. From case 1 three hybrids were selected because of high Ig secretion and were cloned by limiting dilution. The highest secretor (1B9/F2) was used to purify Id from supernatant by affinity chromatography. Purified Id was then used as an immunogen, and two mouse monoclonal anti-Id antibodies (4A10, 3C8) were eventually isolated that reacted with the patient’s Id protein and not Id protein from unrelated B cell tumors. In case 2 individual hybrids were pooled before Id was purified from culture supernatant. One anti-Id antibody (2C1) was isolated.

A cell line was also made from each patient that shared the phenotypic and genotypic characteristics of their original tumor (case 1, SUP-B8; case 2, SUP-B-12). In both cases the anti-Ids reacted specifically with the tumor cell line as well as the respective tumor harvested at diagnosis or relapse. Thus the cell lines were truly derived from the tumor in each case. As an example, Fig 1 demonstrates MAB staining (4A10) on both the original tumor and the cell line from patient 1.

**Serum Id Assay**

Anti-Id antibodies were used retrospectively in an ELISA assay to measure Id in serum samples. In case 1 Id was present in very high levels (1,290 μg/mL) at diagnosis (Fig 2). With the institution of chemotherapy the level fell with the expected half-life of human IgM (5 days) to background.
was no CNS disease was present, there disease was isolated and not associated with uncontrolled the serum. CSF was examined on and no Id was present in a three-week period showed no detectable Id. Although active certain why fluorescence with anti-ti is brighter on the bone anti-AL recognize a major portion of both samples, although it is not.

Panel B, bone marrow from diagnosis case 1

isothiocyanate-conjugated goat antimouse lg (Tago). Irrelevant incubated with anti-Id MAB or a monoclonal mouse antihuman immunofluorescence was used to label the cells. Cells were displayed on the X axis and cell number on the V axis. Indirect immunofluorescence was used to label the cells. Cells were incubated with anti-Id MAB or a monoclonal mouse antihuman µ (1D12) supernatant. The second step consisted of fluorescein isothiocyanate-conjugated goat antimouse Ig (Tago). Irrelevant mouse antibody was used as a control. Panel A, cell line SUP-B8.

Fig 1. FACS histogram of tumor cell labeling. Log fluorescence is displayed on the X axis and cell number on the Y axis. Indirect immunofluorescence was used to label the cells. Cells were incubated with anti-Id MAB or a monoclonal mouse antihuman µ (1D12) supernatant. The second step consisted of fluorescein isothiocyanate-conjugated goat antimouse Ig (Tago). Irrelevant mouse antibody was used as a control. Panel A, cell line SUP-B8. Panel B, bone marrow from diagnosis case 1. Both anti-Id MAB and anti-µ recognize a major portion of both samples, although it is not certain why fluorescence with anti-µ is brighter on the bone marrow sample.

Fig 2. Serum Id levels in case 1 throughout the clinical course. Id was quantitated by a three-step ELISA assay (anti-Id MAB, serum, goat antihuman λ) and background was subtracted by using a third step of goat antihuman x. Id levels fell with chemotherapy. reached background despite the presence of active CNS disease, began to rise slowly, and then increased dramatically before BMT. Asterisks denote when bone marrow was harvested for Southern blot analysis.

Patient 2 also had very high levels of Id at diagnosis (10.8 mg/mL). Four weeks later it had dropped to 680 µg/mL. Ten months later it was zero, consistent with his remission status. However, one month before completing chemotherapy Id was again detected. Id levels initially seemed to increase somewhat, but they have remained relatively stable over the last nine months. Levels were not significantly different using goat antihuman µ or goat antihuman λ as a detector (data not shown), further indicating that a monoclonal protein was being detected. Despite the presence of detectable levels of idiotype, he remains in complete remission without physical or hematologic evidence of recurrent disease.

Fig 3. Serum Id levels in case 2. Very high levels were present at diagnosis, and these fell with chemotherapy. Further serum samples were not available until ten months into treatment, when serum Id was not detected. Just before finishing treatment it was again detected, increased somewhat, and has remained relatively stable for nine months.

Ig Gene Rearrangement

In an effort to compare the sensitivity of assays for serum Id as a tumor marker with other available techniques, bone marrow DNA was analyzed for Ig heavy-chain gene rearrangement. A clonal population of malignant cells of greater than 1% would be expected to result in a discrete band on Southern Blot. DNA was extracted from two bone marrow samples from case 1 taken at a time when Id was first detected in the serum (Fig 3). DNA from tumor at diagnosis and from the Id-secreting hybrid used to generate the anti-Id were also harvested. Figure 4 shows Southern blot analysis of heavy-chain gene sequences. At diagnosis two rearrangements were seen (lane 1), and both of these bands are also present in DNA from the heteromyeloma (lane 2). In addition, the Id-secreting hybrid also contains a 9.5-kb band representing a nonfunctional rearrangement known to be present in the myeloma parent K6H6/B5.6 No rearrangements were detected in two bone marrow samples taken during clinical remission, although Id was detected in the serum concurrently. Thus in this example serum Id appeared to be a more sensitive marker of residual disease.

DISCUSSION

B ALL cells share identical morphological, cytochemical, and cytogenetic features with malignant cells from patients with Burkitt's lymphoma.3,15 Therefore many investigators consider B cell ALL to be an advanced stage of Burkitt's lymphoma.16 Considerable insight was gained into the under-
Fig 4. Ten micrograms of DNA was loaded per lane on an 0.8% agarose gel. Specimens were transferred to an activated nylon filter and probed with a 6.5-kb DNA fragment homologous to the Jκ region of heavy chain. Lane 1, tumor DNA at diagnosis. Two rearrangements are seen (15.2 kb and 8.0 kb). In addition, an 18-kb germine band and a 25-kb crosshybridizing band are seen (arrows). The latter two bands are from contaminating background cells present in the sample. Lane 2, DNA from the Id-secreting hybrid. The 15.2-kb fragment is again seen and the 8.0-kb band is greatly diminished in comparison. This suggests that the upper band is the functioning gene responsible for Ig secretion and the nonfunctional rearrangement has been lost by some hybrid cells. Lanes 3 and 4, DNA from morphologically uninvolved bone marrow taken at a time when Id was redetected in the serum. Only germine bands are seen. Bars represent mol wt standards corresponding to 23.1-kb, 9.4-kb, and 6.6-kb fragments of Hind III digested phage λ DNA.

Understanding of malignant disease when these tumors were shown to be monoclonal in origin by the presence of a single light chain (κ or λ) on the surface of cells from individual tumors and by analysis of different enzyme alleles. Since a clone of B cells is committed to produce only one antibody species, its idiotype is unique to the tumor population and is not present on normal B cells. Our laboratory and others have made murine anti-Id MAB directed against this tumor-specific target to study the biology of B cell malignancies. In addition these reagents have shown promise therapeutically when infused in vivo.

We wished to extend our analysis of adult B cell malignancies to other B cell tumors, specifically Burkitt's tumors. Since these tumors are known to secrete surface Ig into the serum, Id may be a very sensitive biological marker of tumor burden. We report here the development of anti-Id MAB against tumor-specific Id in two patients with B cell ALL. These anti-Id MAB were then used to detect serum Id in both patients. At diagnosis high levels of Id were present in the serum in both cases and indicated that at least 50% of the serum IgM at diagnosis in these patients may be derived from their tumor clone. The values obtained at diagnosis in these two patients (1.2 mg/mL and 10.8 mg/mL) were two to 20 times the value we have noted in adult patients with low-grade B cell malignancies. Two explanations are possible for the high level of Id in these patients. It may reflect the secretion of large quantities of Id by these cells, in agreement with in vitro data from established cell lines. Indeed, the cell lines from the cases reported here secrete up to 1 to 2 μg/mL of Ig in spent supernatant (W. Carroll, unpublished observation, Dec 1986, April 1987). High levels of Ig secretion may also indicate that these cells are arrested at a transformed B lymphocyte stage, as has been suggested by other investigators. Alternatively, or in addition, large amounts of Id may be released from dying cells, since it is well known that this tumor has a high growth fraction with a large portion of cells dying spontaneously. In case 2 the initial Id level (10.8 mg/dL) is ten times the normal IgM level and was determined ten days after diagnosis at a time when the patient displayed serious acute tumor lysis necessitating intensive medical support. Since it is known that these patients usually have normal-to-low total IgM values, the high level reported here is probably due to the rapid lysis of cells (with release of intracellular Ig) due to chemotherapeutic treatment.

Serum Id levels proved to be an excellent measure of tumor burden in patient 1; with the institution of chemotherapy, levels fell to control values and correlated with tumor regression. Small amounts of Id were redetected in the serum seven weeks after its first documented disappearance. Five weeks later it was also detected, and 2½ weeks thereafter it appeared to be increasing in logarithmic proportions. This rapid rise was initially blunted and actually fell somewhat with the preparative regimen used for BMT. However, two samples taken one week apart again demonstrated a logarithmic increase, and leukemic cells were finally detected in the bone marrow (BM) and peripheral circulation one month after transplantation. In this case systemic relapse was predicted well before clinical documentation. Additionally, redetected serum Id levels initially followed a Gompertzian curve with a latent period followed by a phase of logarithmic growth prior to BMT. During this period the Id doubling time was 110 hours, which corresponds to tumor doubling times measured by other methods in vivo and in vitro.

Idiotype was also detected in the CSF in patient 1 after a documented CNS relapse. Residual declining Id was also identified in the serum during the first episode. However, during a second CNS relapse serum Id on three occasions was not detected, implying good control of systemic disease and that recurrent disease was indeed isolated to the CNS. One interpretation would be that systemic disease was well treated and that eventual systemic relapse was due to reseeding from the CNS. This agrees with the known high CNS involvement in this disease and the recognition that better neuroprophylaxis is necessary.

In contrast to patient 1, the reappearance of serum Id did not predict incipient relapse in the second patient. Similar to the first case, the high serum Id detected at diagnosis fell with the institution of chemotherapy and appeared to correlate with tumor burden. However, idiotype was again detected in the serum just prior to completing chemotherapy and has remained elevated during 15 additional months of follow-up without other evidence of leukemic relapse. Since it is unusual for such patients to relapse after 12 months of...
continuous complete remission, the clinical significance of persistent Id is uncertain at the present time. The stable Id level implies tumor dormancy or endogenous regulation between host and malignancy. In this regard we have isolated Id-specific cytotoxic T cells from both patients with B cell ALL reported here. We have not detected Id-positive cells in fixed tissue smears of bone marrow aspirates; and conventional serum protein immunoelectrophoresis has not demonstrated a monoclonal spike (data not shown). However, Id levels currently seen (<100 μg/mL) would be below the level of detection using the latter technique. Conceivably a portion of a host second-order antibody response (Ab2) against endogenous (host) anti-idiotypic (Ab1) could mimic the original antigen (Id, Ab0) and thus be detected by our anti-Id MAB. Such a physiologic role for anti-Id antibodies has never been demonstrated previously. Alternatively an antibody response against an unrelated antigen may be associated with a crossreactive idiotype with the original tumor Ig. Evidence against the latter explanation is that the response reported here is highly restricted. It is limited to a single light-chain (λ) and heavy-chain (κ) isotype, implying a monoclonal proliferation. The lack of a γ component is uncharacteristic for an antigen-driven immune response.

Despite the present uncertainty associated with the reappearance of serum Id in patient 2, serum Id should prove to be a clinically useful measure of minimal residual disease. Response to chemotherapy can be addressed even in the absence of clinically detectable disease (ie, response to chemotherapy, TBI in patient 1 prior to BMT). In addition, the timing of consolidative measures such as BMT could be performed at a time when they would be expected to be most useful when there is considerable "depth" of remission with no serum Id detectable.

In an effort to compare the sensitivity of our assay for the detection of minimal residual disease with other sensitive methods, Southern blot analysis of Ig genes was performed on two BM specimens from case 1, obtained when Id was first detected in the serum. Other investigators have estimated that one leukemic cell in 500 normal cells can be identified using this technique. We were unable to demonstrate the presence of a clonal rearrangement in either sample. Thus in this case Id expression seems to be a much more sensitive marker of residual leukemia. One limitation of Ig gene rearrangement studies would be the irregular involvement of bone marrow by leukemic cells and therefore sampling error. This is a problem shared by other experimental efforts to detect residual tumor cells, including in vitro colony formation.

The presence of monoclonal serum immunoglobulin in Burkitt’s tumors and undifferentiated lymphomas has been studied by other investigators using immunoelectrophoresis. Sera from 15 patients with B cell ALL were studied by Preud’homme et al for the presence of monoclonal Ig. Monoclonal IgM was detected in five cases, and a Bene Jones protein was found in two others. More recently Magrath et al., using a more sensitive electrophorography method, examined patients with undifferentiated lymphomas of Burkitt’s and non-Burkitt’s types. Monoclonal Ig bands were seen in 13 of 31 cases of American undifferentiated lymphoma, and their presence correlated with disease stage. In contrast, no bands were seen in sera from patients with African Burkitt’s lymphoma, and this agrees with the absence of Ig secretion by cell lines established from such patients in tissue culture.

The high levels of Id in patients with B cell ALL would be a serious obstacle to in vivo therapeutic use of these antibodies. Infused MAB would be blocked by secreted Id before reaching their tumor target. Two potential approaches to circumvent this problem would be (1) plasmapheresis, since IgM antibodies (Id) would be limited to the vascular space, and/or (2) chemotherapy to lower tumor burden and thus Id to levels that would allow treatment with anti-Id MAB. However, the biggest limitation to the therapeutic use of such tailor-made antibodies is the time required for their development. At best six to eight months is needed to make anti-Id MAB in quantities sufficient for in vivo therapy. In general, patients with B cell ALL tend to relapse early using conventional chemotherapy (<6 months from diagnosis), and relapses after this time are rare. Therefore by the time MAB is ready for therapeutic use the patient has already relapsed or is likely to be cured. Indeed, patient 1 died just prior to the availability of therapeutic antibody because of the long time necessary for antibody development, and patient 2 has not relapsed in two years following his diagnosis.

In summary, we report the development of anti-Id MAB for two patients with B cell ALL. Serum Id levels were exceedingly high at diagnosis in both cases, and this finding may indicate that the precursor cell of this tumor may be a relatively mature B cell capable of antibody secretion. Id levels very accurately reflected tumor burden and indicated that a considerable depth of remission was initially achieved in both cases. Recurrent disease was detected four months before conventional techniques disclosed the return of leukemic cells in the bone marrow in case 1.

In contrast, the reappearance of serum Id did not predict incipient relapse in patient 2, and the clinical significance of this finding is uncertain at the present time. The data reported here suggest that Id may be one of the most sensitive tumor markers known but that its return after initial complete remission may not necessarily predict imminent relapse.

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