

IDIOTYPE VARIANT CELL POPULATIONS IN PATIENTS WITH B CELL LYMPHOMA

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Idiotype (Id) is an excellent tumor-specific marker for those tumor cells that synthesize and display immunoglobulin (Ig) on their surface (1, 2). Because a given clone of B cells is committed to producing only one Ig species, surface Id is shared by members of the tumor cell population but not by normal cells. We and others have developed murine antiidiotype mAb to study the biology of these diseases, and these reagents have shown some success in the treatment of patients with B cell malignancies (3–6).

However, patients have been recently described whose tumors have developed variant Id proteins spontaneously over time or in response to treatment with mAb (7, 8). A major mechanism of such variation appears to be somatic mutation in genes coding for the variable region of the expressed Ig molecule (9). The discovery of such variant forms of Id raises many issues related to tumor biology and to the potential therapeutic application of mAb. For example, what is the incidence and degree of Id diversity that could occur in B cell tumors? What is the role of potentially mutagenic agents such as chemotherapy and irradiation in generating Id diversity? It is important to define further the natural history of this process by examining tumor populations in vivo over time and, ultimately, to dissect the molecular mechanism responsible for such diversity. In this article we report recent work from our laboratory that examined some of the issues raised by our earlier studies and we describe an approach whereby clonal isolates of the tumor population and their Ig protein products can be obtained and separately analyzed.

Materials and Methods

Patients

Case 1. A 42-yr-old female developed generalized lymphadenopathy after a 1-yr history of lymphocytosis. Bone marrow biopsy results revealed gross lymphomatous involvement and biopsy findings of a right inguinal lymph node were consistent with nodular mixed lymphocytic and histiocytic lymphoma. The clinical stage was IVA. A separate biopsy was performed to obtain tissue for mAb production. No therapy was instituted. Over the next year she experienced spontaneous regression of her disease with resolution of cervical and supraclavicular, lymphadenopathy, although moderate inguinal adenopathy remained. Repeat inguinal lymph node biopsies were performed 1 yr after referral and revealed persistent disease. During the ensuing year disease progressed in multiple lymphatic sites, requiring chemotherapy.

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Case 2. A 50-yr-old female had an epigastric mass, discovered on routine examination. An exploratory laparotomy was performed and a large mesenteric mass was biopsied and disclosed nodular lymphoma, poorly differentiated, clinical stage IIA. Treatment was initiated with chlorambucil and a complete tumor response occurred. This medication was stopped after a total of 1 yr. Four months later, left inguinal adenopathy was noted and a biopsy again showed nodular, poorly differentiated lymphoma. Over the next year, bilateral inguino-femoral adenopathy developed and moderately prominent retroperitoneal nodes were detected by computed tomography scanning. Chemotherapy was reinstated with cyclophosphamide, vincristine, and prednisone, which induced a partial tumor regression. A repeat biopsy was performed and tumor cells were obtained for mAb development. Symptomatic inguino-femoral adenopathy was treated with radiation therapy, although it did not resolve completely. During the following year, cervical, axillary, and epithochlear adenopathy developed, and a repeat biopsy of an axillary node was performed. A subsequent remission was induced by several months of combination therapy consisting of cyclophosphamide, vincristine, procarbazine, and prednisone.

Human Malignant Cells

Tumor samples were minced, passed through a sterile seive, and washed in complete media containing RPMI 1640 (Irvine Scientific, Woodland, CA), with 12% FCS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (Gibco, Grand Island, NY). Samples were frozen in 90% FCS/10% DMSO in liquid nitrogen. After thawing, cells were washed in complete media, and viable mononuclear cells were isolated by sedimentation on a Ficol-Hypaque gradient.

Recovery of Tumor Idiotype

Idiotype was recovered by "rescue fusion" from tumor cells by methods described previously (10, 11). Briefly, tumor cells were fused with K6H6/B5 a mouse human hybrid myeloma cell which is a nonsecretor of Ig. Hybrids were screened for the production of human Ig by an ELISA. Plastic microtiter plates (Immulon 2, Dynatech Diagnostics, Inc., South Windham, ME) were coated with goat anti-human Ig antibody (Tago Inc., Burlingame, CA) at a concentration of 10 µg/ml. Plates were washed with 0.15 M NaCl, 0.05% Triton X-100. 30 µl of supernatant from wells with hybrids was then added and incubated for 1 h at room temperature (RT).¹ The plates were washed and human Ig was detected with goat anti-human µ, κ, and λ antibodies labeled with horseradish peroxidase (HRP) (Tago Inc.) diluted in PBS with 0.1% BSA. Plates were again incubated at RT for 1 h, and enzyme substrate 2,2-azino-di(3-ethylbenzthiazoline sulfonic acid) 150 µg/ml (Sigma Chemical Co., St. Louis, MO) in 0.05 M citrate buffer, pH 4.0, with 0.0045% hydrogen peroxide was added. Color development was measured by an ELISA spectrophotometer at a wavelength of 405 nm (automated micro-ELISA reader, Dynatech Diagnostics, Inc.). Wells with values >20% of positive control (purified human paraprotein 500 ng/well) after subtraction of background were scored as positive.

Wells with the most positive signals were expanded and cloned by limiting dilution using irradiated BALB/c mouse spleen cells as a feeder layer.

Anti-Id Antibodies

Idiotype protein was isolated from culture supernatant by affinity chromatography using goat anti-human µ antibody (Tago Inc.) coupled to a CNBr-activated Sepharose 4B affinity column (Pharmacia Fine Chemicals, Piscataway, NJ). BALB/c mice were immunized i.p. with 50 µg of purified protein in CFA, received a booster 7 d later, and another dose intravenously on day 14. Spleens were removed on day 17 and were fused to P3 × 63 Ag 8.653, a nonsecreting mouse myeloma line, by standard techniques (12). Hybrids were screened for reactivity with purified protein by an ELISA. Id was used to coat microtiter plates (10 µg/ml). Plates were washed and 30–50 µl of culture supernatant from wells with hybrids was added. Plates were incubated for 1 h at RT and were again washed. Purified goat anti-mouse Ig coupled to HRP was then added and incubated for

¹ Abbreviations used in this paper: HRP, horseradish peroxidase; RT, room temperature.

1 h. After washing, substrate was added and color development was measured by methods described above. Positive control consisted of culture supernatant from a hybrid-secreting antibody specific for human μ constant region (1D12) and the negative control was a mAb specific for Id from an unrelated B cell lymphoma.

In order to detect antibodies directed against Ig idiotypic determinants, hybrids that demonstrated reactivity by the above assay were then screened by ELISA on a panel of three Ig proteins from unrelated B cell tumors. Antibodies that retained their specificity were used to stain frozen tissue sections of the patient's original lymphoma by the immunoperoxidase technique (13). Specificity was again checked by staining normal human tonsil. Finally, antibodies were also screened for binding to whole tumor cells from the original tumor by indirect immunofluorescence. An affinity-purified goat anti-mouse Ig reagent coupled to FITC (Becton, Dickinson & Co., Mountain View, CA) was used as a second step. Surface immunoglobulin was detected by direct immunofluorescence using affinity-purified FITC-conjugated antibodies specific for μ , κ , and λ chains (Becton, Dickinson & Co.) or by indirect immunofluorescence using a mouse monoclonal antibody specific for the μ constant region (1D12) followed by the FITC-labeled anti-mouse reagent. T cells were detected by staining with FITC-conjugated human anti-Leu-4 mAb (Becton, Dickinson & Co.). All fluorescence analysis was performed with a fluorescence-activated cell sorter (FACS IV, Becton, Dickinson & Co.).

Competitive Inhibition Assays

Purified Id (case 1) was used to coat flexible microtiter plates (Becton, Dickinson & Co.) at a concentration of 10 $\mu\text{g/ml}$ at 4°C overnight. Anti-Id antibodies were purified from supernatant over a CNBr-activated Sepharose 4B column (Pharmacia Fine Chemicals) to which a goat anti-mouse antibody (Tago Inc.) had been coupled. Antibodies were iodinated with ^{125}I by the chloramine T method (14). Plates were washed and blocked with 2.0% nonfat dry milk in PBS for 45 min at RT. After rewashing, 50 μl of anti-Id mAb supernatant was added and incubated at RT for 1 h. All supernatants were adjusted with complete media to the concentration present in the lowest secretor (1.5 $\mu\text{g/ml}$). ^{125}I -labeled antibody was then added and incubated for an additional 30 min. Plates were washed and dried, and individual wells were counted. Each antibody was tested for its ability to block itself and the four other mAb. Maximum binding was assessed by using an irrelevant mAb directed against Id from an unrelated B cell tumor as a first step. Each assay was done in quadruplicate and percent inhibition was defined as % inhibition = $100 \times (1 - \text{experimental cpm}) / (\text{maximum cpm})$.

Analysis of Id-producing Hybrids with Anti-Id Antibodies

Rescue hybrids from case 1 that secreted intact Ig were tested further for their reactivity with a panel of anti-Id mAb. A four-step ELISA assay was used: goat anti-human Ig, culture supernatant, anti-Id supernatant, and goat anti-mouse Ig (Tago Inc.). Positive control again consisted of a mouse anti-human μ reagent (1D12) and negative control consisted of supernatant alone. Wells with signals at least 0.100 OD units above control after subtraction of background were scored as positive.

Southern Blot Analysis of Rearranged Ig Genes

DNA was isolated from whole tumor samples, the myeloma parent line K6H6/B5, and Id-producing rescue hybrids. 10 μg of DNA was digested with the appropriate restriction enzyme (New England Biolabs, Beverly, MA) overnight at 37°C, and samples were then electrophoresed on a 0.8% agarose gel. DNA was transferred to an activated nylon filter (Genatran 45, Plasco Inc., Woburn, MA), and filters were probed with ^{32}P -labeled genomic sequences homologous to the J_H and C_μ regions of the heavy chain and to C_λ regions of the light chain by methods described previously (11, 15, 16). The heavy-chain probes were a 6.5 kb Bam HI-Hind III fragment, which includes the germline J_H region and a 1.2 kb Eco RI fragment, which spans the first, second, and a portion of the third exon of the C_μ gene (17). A combined C_λ probe consisting of a 2.5 and a 3.5 kb Eco RI-Hind III fragment was also used (18). Phage λ DNA digested with Hind III was run in parallel as a molecular size standard.

Results

Analysis of Tumor with Anti-Id Antibodies. One idiotype protein that was the product of a cloned rescue hybrid from each patient's tumor was purified, and five independent anti-Id mAb were generated for each patient. These were used to analyze tumor samples by immunoperoxidase on tissue sections, and by immunofluorescence on cell suspensions. Results of tissue section staining from both patients showed that different portions of tumor were recognized by individual mAb (Fig. 1). Certain mAb seemed to recognize all of the tumor cells defined by surface μ staining, whereas others bound to smaller subsets of cells. Populations of tumor stained by individual mAb were overlapping and not totally distinct from one another.

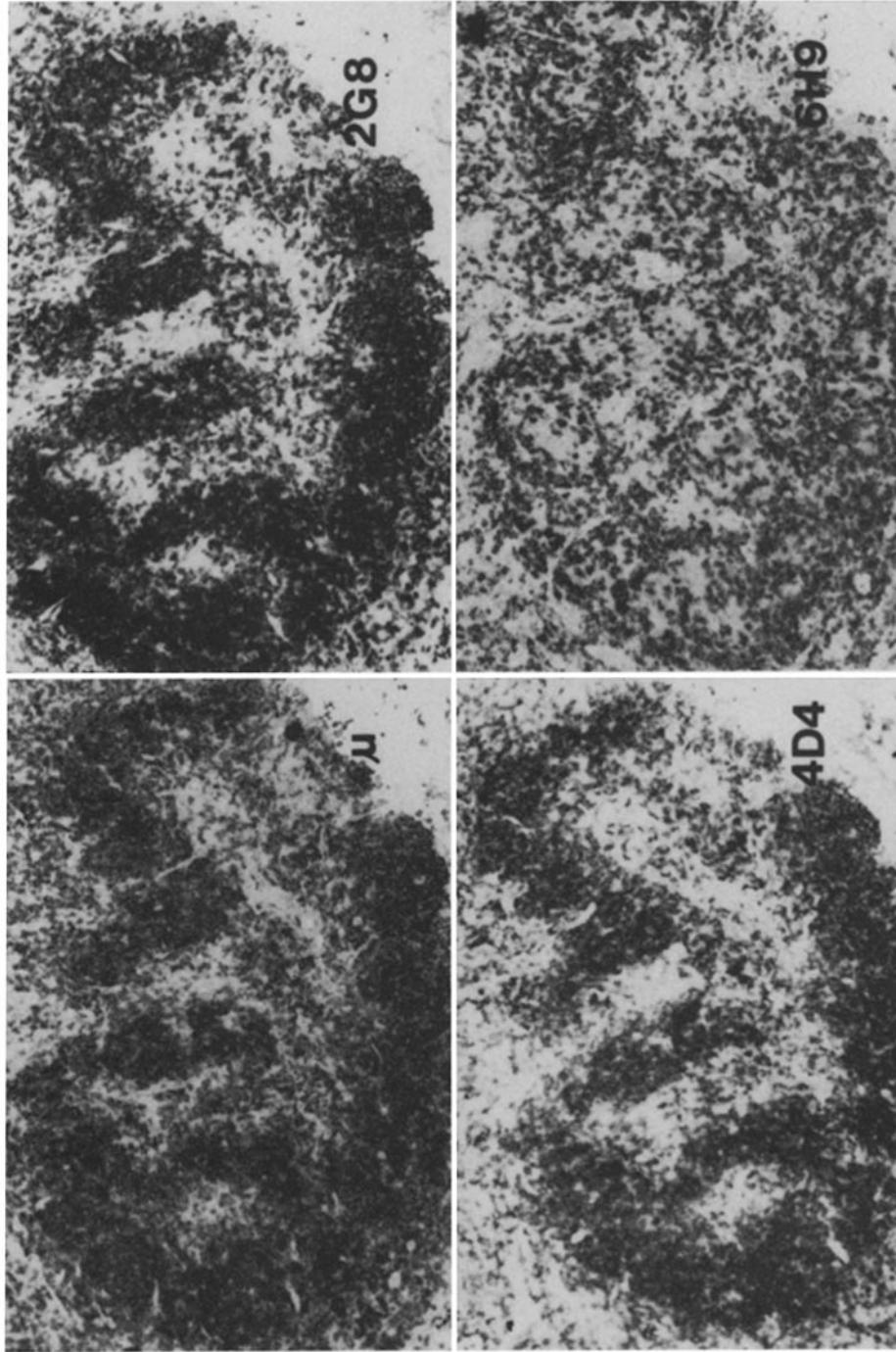
In order to quantitate this phenomenon better, FACS analysis of tumor cell suspensions was performed. Table I shows staining of biopsy material from both cases taken at the time of referral and another sample harvested 1 yr later. Samples were analyzed for T cell infiltration using anti-Leu-4, and total tumor was again estimated by surface μ staining or by subtracting the percentage of cells stained for the irrelevant light chain from those bearing the light chain associated with tumor ($\lambda - \kappa$). The five mAb were again tested for their reactivity with the tumor. It is apparent that these antibodies recognized subpopulations of total tumor and that no single mAb stained the total tumor, although a few bound the majority of cells (case 1, 2G8, 4D4; case 2, 5D3). However, a mixture of all five antibodies captured total tumor in some instances (case 1, 1983; case 2, 1983). The small difference between the percentage of tumor cells detected by the mixture of antibodies in case 2 one year after referral might represent infiltration with normal B cells. Alternatively, it is possible that an increasing number of Id variants had evolved during the year.

The percentage of tumor recognized by a single antibody could differ between tumor samples taken from different anatomical locations. For example, antibody 3F2 recognized 16% of cells taken from one inguinal lymph node in patient 1 and captured twice as many, 34%, when analyzed on tumor taken from an adjacent lymph node sampled at the same time. Percent total tumor was similar in both lymph nodes. In addition, these percentages could change over time. Antibody 1D9 recognized 38% of cells from a lymph node from patient 2 at referral and 62% of the cells were stained 1 yr later.

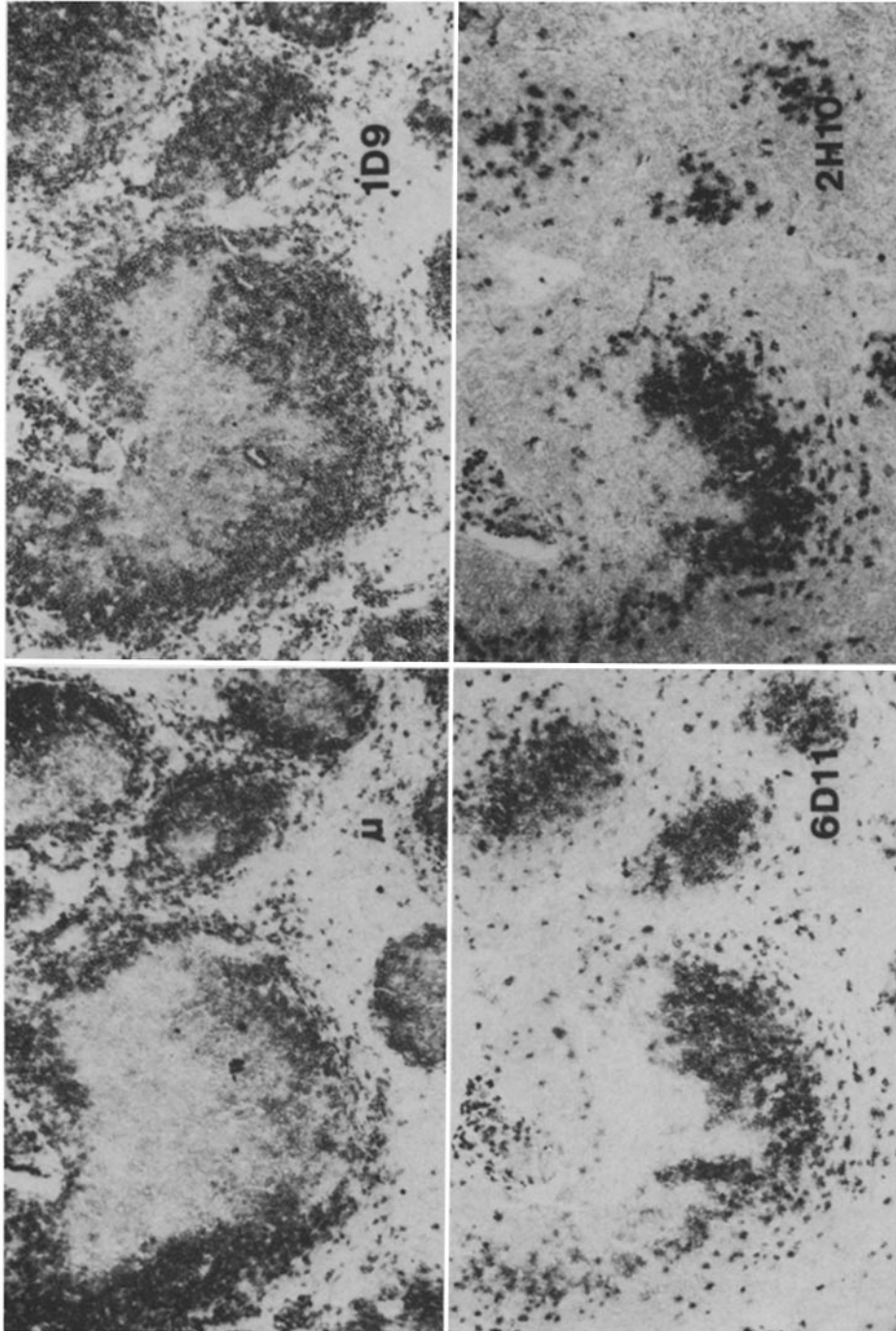
Because a single cloned rescue hybrid was used as a source of Id protein to create anti-Id antibodies in both these cases, it would appear that each anti-Id recognized a distinct antigenic site (idiotope) on the variable region of the molecule. These idiotopes were variably shared by the Ig molecules produced by the tumor population.

Competitive Binding Assays. Competitive binding assays were performed to prove that individual anti-Id antibodies recognized different idiotopes as predicted by the staining data. These results summarized in Table II show that no two mAb share an identical blocking pattern. In addition, the data suggest that these idiotopes are closely related spatially, in that the binding of a single antibody (4D4) blocks targeting of all the other anti-Id mAb.

Recovery of Individual Id Variants. Extensive analysis of individual hybrids from case 1 was carried out in order to sort individual Id variants from a heterogeneous tumor sample. Each hybrid would be expected to secrete a single



A



B

TABLE I
Anti-Id Antibodies Recognize Tumor Subpopulations

| | Sample | | T cell Leu-4 | Ig μ | Anti-Id | | | | | |
|--------|--------|--------------|-----------------|--------------------|---------|------|------|-----|-----|-----|
| | Date | Site | | | 3C10 | 6H9 | 4D4 | 2G8 | 3F2 | Mix |
| Case 1 | 1983 | Inguinal | 13* | 75 | 31 | 48 | 45 | 63 | 23 | 76 |
| | 1984a | Inguinal | 33 | 60 | 20 | 35 | ND | 45 | 16 | 54 |
| | 1984b | Inguinal | 33 | 65 | 29 | 43 | ND | 54 | 34 | 52 |
| | | | Leu-4 | $\lambda - \kappa$ | 5D3 | 6D11 | 2H10 | 1D2 | 1D9 | Mix |
| Case 2 | 1983 | Inguinal | 25 | 75 | 60 | 11 | 16 | 11 | 36 | 71 |
| | 1984 | Epitrochlear | 19 | 69 | 61 | 20 | 14 | 5 | 62 | 58 |

* Values are percentage of total sample stained by FACS analysis.

TABLE II
Competitive Binding to Id by Anti-Id Antibodies

| ¹²⁵ I-labeled antibodies | Percent inhibition by unlabeled antibodies | | | | |
|--|--|-----|-----|------|-----|
| | 4D4 | 3F2 | 6H9 | 3C10 | 2G8 |
| 4D4 | 81 | 46 | 66 | 31 | 65 |
| 3F2 | 80 | 77 | 10 | 0 | 73 |
| 6H9 | 62 | 0 | 92 | 45 | 6 |
| 3C10 | 73 | 0 | 76 | 52 | 62 |
| 2G8 | 82 | 60 | 14 | 57 | 93 |

idiotype species. The fusion was initially screened for heavy- and light-chain secretion, and those hybrids that secreted complete Ig were then screened with four of the five anti-Id antibodies. Mathematically, a total of 16 different patterns could be recovered if each antibody recognized a distinct epitope and if a representative of each of the possible combinations existed in the panel of rescue proteins. In fact, 11 different reactivity patterns were observed (Table III). Those not seen would be expected to be present in low frequency based on the percentage of total tumor sample stained by individual mAb (Table I). For example, patterns lacking binding with antibodies 4D4 and 2G8 should be rare because these antibodies reacted with a major portion of the tumor cell population. Strict statistical correlation is confounded somewhat by the possible presence of more than one hybrid per well, inasmuch as supernatants were tested without prior subcloning. 13% of the hybrids produced an Ig product that did not react with any of the four anti-Id antibodies. It is possible that these represent the products of normal B cells within the biopsy specimen. Alternatively, they could

FIGURE 1. (A) Lymph node biopsy obtained from case 1 at presentation. Tissue is stained with mAb by the immunoperoxidase technique. Total tumor is recognized by staining with anti- μ reagent (*upper left*). Three different anti-Id antibodies were then used to stain the same area and this is seen on the succeeding three panels. Anti-Id mAb recognized subpopulations of total tumor. Antibodies 2G8 and 4D4 saw a major portion of tumor whereas 6H9 recognized a smaller fraction. (B) Lymph node biopsy from case 2 at referral. Total tumor is again approximated by surface μ staining (*upper left*). Three anti-Id mAb stained varying portions of total tumor. Antibody 1D9 (*upper right*) seemed to stain total tumor when compared to anti- μ staining (*upper left*). Anti-Id mAb 6D11 and 2H10 recognized much smaller fractions.

TABLE III
Recovery of Id Variants from Heterogeneous Tumor Population

| Predicted patterns | Anti-Id antibodies | | | | Wells positive (n) | Percent positive |
|--------------------|--------------------|-----|-----|-----|--------------------|------------------|
| | 3C10 | 6H9 | 4D4 | 2G8 | | |
| 1 | +* | + | + | + | 158 | 46 |
| 2 | - | - | - | + | 71 | 20.9 |
| 3 | - | - | - | - | 44 | 13.0 |
| 4 | - | - | + | + | 32 | 9.4 |
| 5 | + | - | + | + | 11 | 3.2 |
| 6 | - | - | + | - | 9 | 2.7 |
| 7 | - | + | + | + | 4 | 1.2 |
| 8 | + | + | - | + | 3 | 0.88 |
| 9 | + | - | + | - | 3 | 0.88 |
| 10 | + | + | + | - | 3 | 0.88 |
| 11 | - | + | - | + | 1 | 0.30 |
| 12 | + | - | - | + | 0 | — |
| 13 | + | - | - | - | 0 | — |
| 14 | + | + | - | - | 0 | — |
| 15 | - | + | + | - | 0 | — |
| 16 | - | + | - | - | 0 | — |
| Total: | | | | | 339 | |

* +, reactivity with anti-Id mAb; -, nonreactivity with mAb.

represent tumor variants lacking idiotopes detected by these particular anti-Id antibodies.

Southern Blot Analysis of Ig Genes. The genetic mechanism responsible for such diversity was explored using Southern blot analysis of rearranged Ig genes on DNA obtained from biopsy material and from two hybrids from case 1, each producing an Ig product with a different pattern of reactivity with anti-Id antibodies. The analysis was also performed on the parental myeloma fusion partner used to create these hybrids, because it carries a nonfunctional rearrangement for the heavy chain (11). Using Eco RI-digested material from biopsy material, two heavy-chain rearrangements were detected using a J_H probe (Fig. 2A): one larger (20.1 kb) and one smaller (10.2 kb) than the germline band (represented by the arrow). The active allele is the lower 10.2-kb band (represented by the arrowhead) because it is the only rearrangement seen in Id-secreting hybrid 2 (11). The K6H6/B5 myeloma parent had one rearranged band, and this was retained by hybrid 1 (-+++). In addition, this hybrid demonstrated two smaller rearrangements not seen in the analysis of biopsy material. In contrast, hybrid 2 (-+++) contained a single band, which was identical to the lower rearrangement seen in tumor tissue. The two smaller bands in hybrid 1, when added together, approximated the size of the active rearrangement seen in the biopsy and in hybrid 2. Therefore, it appears that an Eco RI site had been created in the J_H region in the active heavy chain allele retained in hybrid 1 (-+++).

In order to analyze further the diversity seen in the heavy-chain gene, a different enzyme, Bam HI, was used to digest DNA samples and a different probe C_μ, a 1.2-kb germline sequence that hybridizes to a portion of the constant region of the heavy chain was used. Tumor tissue was analyzed previously (data not shown) and the location of the productive rearrangement seen in biopsy

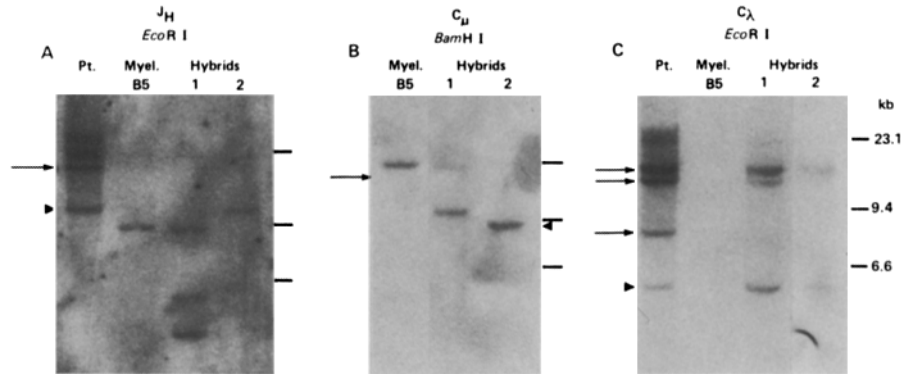


FIGURE 2. Southern blot analysis of rearranged Ig genes. Tumor DNA was obtained from a lymph node biopsy taken from case 1 at diagnosis. DNA was also harvested from two rescue hybrids generated from a fusion between tumor (also obtained from case 1 at diagnosis) and K6H6/B5 a nonsecreting mouse human heteromyeloma cell line. The Id protein secreted by each of these hybrids demonstrated a different pattern of reactivity with four of five anti-Id mAb (Table III). Hybrid 1 Id was recognized by three of four mAb (---+++), while hybrid 2 was bound by two (---++). DNA from the myeloma fusion partner was also run for comparison because it is known to contain a nonfunctional rearrangement (11). Two probes were used to identify heavy-chain sequences: a 6.5-kb Bam HI/Hind III sequence which includes the entire J_H germline region, and a 1.2 kb Eco RI C_μ probe which spans the first, second, and a portion of the third exon of the C_μ germline sequence (17). Two germline sequences were combined and used to detect light-chain rearrangements, a 2.5-kb Eco RI-Hind III fragment, which includes the Mcg C_λ sequence and a 3.5-kb Eco RI-Hind III fragment containing the Ke⁻Oz⁻ C_λ gene. DNA from normal granulocytes was used to identify germline sequences and these are indicated by *arrows*. Molecular weight standards corresponding to Hind III-digested phage λ DNA are also indicated. (A) Eco RI digestion, J_H probe. Two rearrangements were seen with biopsy material (20.1 and 10.2 kb). The active rearrangement is the lower 10.2-kb fragment in that it is the only rearrangement retained by Id-secreting hybrid 2 (11) and this is identified by an *arrowhead*. In addition, a third larger band is seen and this represents a partially digested fragment because it was not seen on multiple blots done previously using the same sample, enzyme digestion, and probe. The K6H6/B5 myeloma parent contains a band and this was retained in hybrid 1. Hybrid 2 retained a band that is identical to the active rearrangement seen with biopsy material. However, hybrid 1 demonstrated two smaller fragments whose size when added together approximated the size of the active rearrangement. (B) Bam HI digestion, C_μ probe. Biopsy material was run previously (data not shown) and the location of the active rearrangement is indicated by the *arrowhead* (11). The myeloma parent again demonstrated a single nonproductive rearrangement and this was again seen in hybrid 1. Hybrid 1 also contained a 10.9-kb rearrangement while hybrid 2 showed the same 9.5-kb band seen in biopsy material. Thus, Southern blot analysis with two different enzyme digestions (combined with two heavy-chain gene probes) demonstrated different restriction patterns in the rearranged genes coding for Id proteins with differential staining by a panel of anti-Id mAb. (C) Analysis of light-chain rearrangements using Eco RI digestion and C_λ probe. Three germline bands were seen in biopsy material and a larger 22.8-kb band was also seen in tumor and control DNA. An identical 6.0-kb rearrangement was seen in the tumor tissue and both hybrids. Therefore, in contrast to differences in heavy-chain sequences both hybrids have identical light-chain rearrangements.

material is indicated by the triangle seen in Fig. 2B (11). Again, hybrid 2 shared an identical 9.4-kb rearrangement with that seen in biopsy material and a dissimilar larger (10.9 kb) rearrangement was seen in hybrid 1. This suggests that a Bam HI site had been lost within the gene coding for this Id variant. Again, the myeloma parent contained a nonfunctional rearrangement and this is represented by a band in hybrid 1.

In contrast to the diversity seen in analysis of heavy-chain genes, the λ light chains, within the limits of analysis using a single enzyme and single probe, were

identical. Three germline bands are seen in the biopsy sample (Fig. 2C) and a larger (23 kb) band was also present in both tumor and control DNA. This most likely represents a restriction site polymorphism known to be associated with the lower germline band. (19) One 6.0-kb, representing the active rearrangement, is seen in biopsy material and this is shared by both hybrids.

Discussion

Most human lymphoid neoplasms are of B cell lineage, and these diseases mirror various stages of normal B cell development (20). Because each B cell is programmed to produce only one antibody species, those malignancies that express surface Ig provide an opportunity to specifically mark tumor cells with tailor-made antibodies. We have used anti-Id antibodies to study tumor biology in this group of diseases and have explored their therapeutic use (3, 5, 6, 12). Indeed, these reagents have shown promise in clinical trials, with 50% of patients achieving a clinically significant response (6). Although one sustained complete clinical remission has been achieved, most of these responses have been short in duration, with eventual regrowth of tumor.

One potential mechanism of tumor escape is through the development of Id variants within the tumor population that cease to bind antibody. If the effects of antibody were powerful, and if subpopulations of Id variants existed within the tumor population, then treatment with a single mAb would be expected to provide a strong selective advantage to the variants not sharing the antibody-binding site (idiotope) recognized by that mAb. Recently, we have observed this exact occurrence (7).

The mechanisms responsible for the enormous antibody diversity achieved by the immune system are better understood. A series of germline DNA segments, three for the heavy chain (V, D, J) and two for the light chain (V, J), rearrange to form one functional coding sequence for the variable region of each Ig chain (21–23). Combinational possibilities owing to the selection of one out of several hundred V segments with one of a series of D segments (heavy chain) and a small number of J segments result in enormous diversity. In addition, the exact site at which these different regions combine is imprecise, and nucleotides may be added at the D segment boundaries, thus amplifying the process still further (24–27). Finally, pairing of different light and heavy chains accounts for another level of antibody diversity.

In addition to the above mechanisms, it is now generally accepted that point mutations within the rearranged variable region encoding sequences serve to expand enormously the antibody repertoire, especially during the primary immune response (28–30). Indeed, the role of somatic mutation in active Ig V region genes of normal B cells has been estimated to be 10^{-3} per base pair per cell division (31). Most of this information on somatic mutation comes from the study of murine myelomas and hybridomas with a restricted antibody response against simple haptens, and little information is available regarding the role of somatic mutation in the human immune response. In that B cell tumors represent stages of normal B cell development, they may be expected to undergo some of the processes leading to antibody diversity seen in the normal immune response.

We have previously described cases of antibody (Id) diversity within B cell tumors whereby subclones emerged over time or after treatment with mAb and

did not bind the single mAb (7). In these cases Id⁺ and Id⁻ populations shared identical Ig gene rearrangements, and the changes appeared to be the result of point mutation within common rearranged V(D)J sequences. This allowed for a structural change in the idiotope recognized by the anti-Id antibody (7). We have recently documented the role of somatic mutation in generating Id diversity at the nucleic level in one of these cases (9).

In this paper we report that striking variations of Id structure may exist at the time of diagnosis in patients with B cell tumors. This extreme diversity was documented by the differential staining of tumor by a panel of anti-Id mAb in both tissue sections and by FACS analysis. Crossblocking studies confirmed that antibodies were directed against different idiotopes on the Ig molecule. In one case, individual variant Id proteins were recovered after fusing tumor tissue with a nonsecreting heteromyeloma line. If each of the resulting hybrids secreted a single species of Id, and the four mAb recognized different Id determinants, then 16 different patterns should emerge. 11 of 16 patterns were actually observed, and the five patterns not observed would be expected to be infrequent. Thus, it appears as though the Id variants secreted by these hybrids mirrors their representation within the original tumor.

These studies also demonstrate that diversity can occur prior to the institution of potentially mutagenic therapy such as chemotherapy or irradiation, although it is possible that such exposure would amplify the apparent high rate of somatic mutation.

In order to generate the panels of mAb described in this report, a single Id protein (from one cloned hybrid) was used as an immunogen, and five different anti-Id mAb were generated. Each of these mAb reacted with a distinct but sometimes overlapping subpopulation of tumor cells. It would appear then that even though extensive tumor Id variation is seen in these cases, these proteins are related with multiple idiotopes being shared by varying portions of the total tumor population. Further evidence of a common origin is seen on Southern blot analysis of rearranged λ genes (case 1), where bands of identity are visualized between tumor and both hybrids. Because these variants are related, it is likely that somatic variation led to the development of multiple forms from a common rearranged V(D)J precursor. Extensive somatic point mutation as occurs in normal B cells would be the most likely genetic mechanism responsible for the observed tumor variability.

Extensive somatic variation is also suggested by Southern blot analysis of rearranged heavy-chain genes. Differences in restriction patterns were seen between two hybrids derived from members of the same tumor clone. By using two different probes (J_H , $C\mu$), hybrid 2 (---++) revealed a band on two different restriction digests (Bam HI, Eco RI) that was identical to that seen in analysis of whole tumor. However, hybrid 1 (-+++)) showed patterns that were consistent with the loss of one restriction site (Bam HI) and the creation of another (Eco RI). The Eco RI site was created within the J_H region, which places this site of mutation within the third hypervariable region of the V protein encoding sequence. The size of the Bam HI restriction fragment (~9.4 kb) would place the site of mutation within the intron separating the variable and constant portions of the Ig coding sequence (17). Thus mutations seem to be taking place in coding and noncoding portions of these genes.

We previously reported cases of tumor Id variations that were discovered when few subclones evolved that lost the ability to bind a single mAb (7). However, when these clones were analyzed at the nucleic acid level, multiple variants with extensive nucleic acid substitutions were identified even before mAb treatment (9). The extreme heterogeneity reported here may be due to even more extensive changes in DNA sequence, or it may be attributed to our ability to detect changes in protein structure more readily, in that multiple mAb were targeted to different idiotopes. Genetic analysis correlating changes in protein structure with nucleic acid substitutions is needed to clarify this issue.

The cases presented here are exceptional in our experience in that a large number of variants were documented. However, it is noteworthy that subpopulations defined by anti-Id antibodies were relatively stable over a long period of time, suggesting that mutation may have occurred early in the evolution of the malignant clone or that certain Id proteins had a selective advantage. In the case previously reported, the somatic mutational process resulted in nonrandom clustering of nucleic acid substitutions along the V_H region (9). More specifically, no changes were noted in CDR1 and CDR3, whereas CDR2 accumulated many mutations, most of which resulted in amino acid substitution. Thus, endogenous selective forces may be active to preserve certain regions while allowing others to change. The nature of such forces is uncertain. Antigen-driven systems have been well described in murine responses to simple haptens, whereby mutations produce antibodies with increased affinity for antigen (30, 32–34). Despite the degree of mutation that we have now seen in B cell tumors, there seems to be a selection for functional Ig molecules. Alternatively, an Id network mediated by either antibody or T cells may be operative.

One obvious question is why the multiple bands seen in hybrid 1 (–+++) were not seen in samples of lymph node biopsies. Based on the percentage of tumor stained by individual mAb (from Table I), the expected frequency of this variant would be on the order of 5%, which would be below the level of detection on these blots.

The recognition of dissimilar restriction patterns in analysis of the heavy-chain genes from different but related variants suggests that Southern blot analysis must be carefully interpreted if it is to be used to identify clonal relationships. Multiple different enzyme and probe combinations are sometimes required, as has been previously shown by others (35). We show here that multiple rearranged bands (more than two) may actually represent extensive somatic mutation within common V(D)J rearrangements rather than the existence of multiple clones (subclones) with different germline V(D)J sequences.

The existence of multiple Id variants raises many issues concerning the potential therapeutic usefulness of anti-Id mAb. It is clear that such mutation occurs to a different degree in every patient, and this may reflect the different stages of B cell differentiation represented by these tumors. In addition, our previous experience in clinical trials indicates that mAb may apply a powerful positive selective force, amplifying Id variants that were not recognized by the mAb. A collection of anti-Id antibodies can clearly be generated that would be targeted to different idiotopes shared by varying numbers of tumor cells. This can be facilitated by using multiple Id proteins rather than the product of a single cloned rescue hybrid as an immunogen to create anti-Id mAb. Further-

more, mAb with more comprehensive tumor reactivity can be selected by screening on multiple Id protein isolates from the tumor. Although an individual Id variant may lose certain idiotopes through somatic mutation, it will retain others that could still allow its detection by the appropriate mAb. It is encouraging to note that in these two cases a single randomly selected Id protein was used to create a panel of mAb, which when pooled together reacted with a majority of tumor, both at diagnosis and 1 yr later. Thus, even in these two cases with striking Id variation (the most striking to date), the majority of tumor, retained idiotypes recognized by mAb. Clinical trials using multiple anti-Id mAb are currently underway.

Summary

Using isolated idiotype (Id) protein we generated panels of antibodies in two patients with follicular lymphoma, one of whom had never received prior chemotherapy or radiotherapy. Flow cytometry and frozen section tissue staining of tumor with these monoclonal antibodies (mAb) revealed multiple subpopulations within each tumor. Individual mAb stained between 7% and 83% of surface Ig⁺ cells in the tumor samples. These subpopulations were overlapping and no single antibody recognized all the tumor cells. However, combinations of antibodies seemed to capture total tumor in both cases. In some instances, the percentage of tumor stained by a single mAb varied over time, and differed between lymph nodes sampled at the same time. Because a single species of Id protein was used to generate mAb in each case, it appears that the antibodies were directed against idiotopes variably shared by different populations within each tumor, and this was confirmed by crossblocking studies. Tumor cells from one patient were fused to a nonsecreting heteromyeloma line K6H6/B5, and most of the resulting hybrids secreted Id protein. Four mAb were used to screen the Id proteins secreted by these hybrids, and 11 different variants (16 maximal) were found. Southern blot analysis of rearranged Ig genes was done in two hybrids and biopsy material. Identically rearranged light-chain genes were seen but it appeared as though extensive somatic variation had occurred in heavy chain genes.

These studies indicate that: (a) striking Id variation can exist at diagnosis in untreated patients, (b) the percentage of tumor represented by an individual variant may change with time and may differ between tumor sampled from different anatomical locations, and (c) somatic variation appears to be responsible for the observed heterogeneity. Although this degree of variation makes anti-Id antibody therapy more difficult, appropriate combinations of mAb should be more efficacious than single antibodies in such cases.

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References

1. Stevenson, G. T., E. V. Elliot, and F. K. Stevenson. 1977. Idiotypic determinants on the surface immunoglobulin of neoplastic lymphocytes: a therapeutic target. *Fed. Proc.* 36:2268.

2. Haughton, G., L. L. Lanier, G. F. Babcock, and M. A. Lynes. 1978. Antigen-induced murine B cell lymphomas. II. Exploitation of the surface idiotype as tumor specific antigen. *J. Immunol.* 121:2358.
3. Brown, S., J. Dilley, and R. Levy. 1980. Immunoglobulin secretion by mouse \times human hybridomas: an approach for the production of anti-idiotype reagents useful in monitoring patients with B cell lymphoma. *J. Immunol.* 125:1037.
4. Mayami, M., H. Kubagawa, G. A. Omura, W. E. Gathings, J. F. Kearney, and M. D. Cooper. 1982. Studies in the clonal origin of B cell leukemia using monoclonal anti-idiotype antibodies. *J. Immunol.* 129:904.
5. Miller, R. A., D. G. Maloney, R. Warnke, and R. Levy. 1982. Treatment of B cell lymphoma with monoclonal anti-idiotype antibody. *N. Engl. J. Med.* 306:517.
6. Meeker, T. C., J. Lowder, D. G. Maloney, R. A. Miller, K. Thielemans, R. Warnke, and R. Levy. 1985. A clinical trial of anti-idiotype therapy for B cell malignancies. *Blood.* 65:1349.
7. Meeker, T., J. Lowder, M. L. Cleary, S. Stewart, R. Warnke, J. Sklar, and R. Levy. 1985. Emergence of idiotype variants during therapy of B cell lymphoma with anti-idiotype antibodies. *N. Engl. J. Med.* 312:1658.
8. Raffeld, M., L. Neekers, D. L. Longo, and J. Crossman. 1985. Spontaneous alteration of idiotype in a monoclonal B cell lymphoma: escape from detection by anti-idiotype. *N. Engl. J. Med.* 312:1653.
9. Cleary, M. L., T. C. Meeker, S. Levy, E. Lee, M. Trela, J. Sklar, and R. Levy. 1986. Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. *Cell.* 44:97.
10. Levy, R., and J. Dilley. 1978. Rescue of immunoglobulin secretion from human neoplastic lymphoid cells by somatic cell hybridization. *Proc. Natl. Acad. Sci. USA.* 75:2411.
11. Carroll, W. L., K. Thielemans, J. Dilley, and R. Levy. 1986. Mouse \times human heterohybridomas as fusion partners with human B cell tumors. *J. Immunol. Methods.* 89:61-72.
12. Thielemans, K., D. G. Maloney, T. Meeker, J. Fujimoto, C. Doss, R. A. Warnke, J. Bindl, J. Gralow, R. A. Miller, and R. Levy. 1984. Strategies for production of monoclonal anti idiotype antibodies against human B cell lymphomas. *J. Immunol.* 133:495.
13. Wood, G. S., and R. A. Warnke. 1982. The immunologic phenotyping of bone marrow biopsies and aspirates: frozen section techniques. *Blood.* 59:913.
14. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131-labeled human growth hormone of high specific activity. *Nature (Lond.).* 194:495.
15. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.
16. Feinberg, A. D., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.
17. Ravetch, J. V., V. Siebenlist, S. Korsmeyer, T. A. Waldmann, and P. Leder. 1981. Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D genes. *Cell.* 27:583.
18. Hieter, P. A., G. F. Hollis, S. J. Korsmeyer, T. A. Waldman, and P. Leder. 1981. Clustered arrangement of immunoglobulin constant region genes in man. *Nature (Lond.).* 294:536.
19. Taub, R. A., G. F. Hollis, P. A. Hieter, S. Korsmeyer, T. A. Waldman, and P. Leder. 1983. Variable amplification of immunoglobulin λ light chain genes in human populations. *Nature (Lond.).* 304:172.
20. Waldman, T. A. 1985. Molecular genetic analyses of human lymphoid neoplasms: immunoglobulin genes and c myc oncogene. *Ann. Intern. Med.* 102:497.

21. Hozumi, N., and S. Tonegawa. 1976. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc. Natl. Acad. Sci. USA* 73:3628.
22. Weigert, M., R. Perry, O. Kelley, T. Hunkapiller, J. Schilling, and L. Hood. 1980. The joining of V and J gene segments creates antibody diversity. *Nature (Lond.)* 283:497.
23. Leder, P., E. E. Max, and J. G. Seidman. 1980. The organization of immunoglobulin genes and the origin of their diversity. In *Immunology 80: Progress in Immunology IV*. M. Fougereau and J. Dausset, editors. Academic Press, Inc., New York. 34-50.
24. Early, P., H. Huang, M. Davis, K. Calame, and L. Hood. 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: V_H, D and J_H. *Cell* 19:981.
25. Max, E. E., J. G. Seidman, and P. Leder. 1979. Sequences of five potential recombination sites encoded close to an immunoglobulin K constant region gene. *Proc. Natl. Acad. Sci. USA* 76:3450.
26. Sakano, H., K. Huppi, G. Heinrich, and S. Tonegawa, 1979: Sequences at the somatic recombination sites of immunoglobulin light chain genes. *Nature (Lond.)* 280:288.
27. Kurosawa, Y., and S. Tonegawa. 1982. Organization, structure, and assembly of immunoglobulin heavy chain diversity DNA segments. *J. Exp. Med.* 155:201.
28. Baltimore, D. 1981. Somatic mutation gains its place among the generators of diversity. *Cell* 26:295.
29. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature (Lond.)* 302:575.
30. Berek, C., G. M. Griffiths, and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. *Nature (Lond.)* 316:412.
31. Teillaud, J., C. Desaynard, A. M. Giusti, B. Haseltine, R. R. Pollock, D. E. Yelton, D. J. Zack, and M. D. Scharff. 1983. Monoclonal antibodies reveals the structural basis of antibody diversity. *Science (Wash. DC)* 222:721.
32. Griffiths, G. M., C. Berek, M. Kaartinen, and C. Milstein. 1984. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. *Nature (Lond.)* 312:271.
33. Clarke, S. H., K. Huppi, D. Ruezinsky, L. Staudt, W. Gerhard, and M. Weigert. 1985. Inter- and intracloonal diversity in the antibody response to influenza hemagglutinin. *J. Exp. Med.* 161:687.
34. Wysocki, L., T. Manser, and M. L. Gelfer. 1986. Somatic evolution of variable region structures during an immune response. *Proc. Natl. Acad. Sci. USA* 83:1847.
35. Siegelman, M. H., M. L. Cleary, R. Warnke, and J. Sklar. 1985. Frequent biclonality and Ig gene alterations among B cell lymphomas that show multiple histologic forms. *J. Exp. Med.* 161:850.