

## STUDIES ON THE CLONAL ORIGIN OF MULTIPLE MYELOMA

### Use of Individually Specific (Idiotype) Antibodies to Trace the Oncogenic Event to its Earliest Point of Expression in B-Cell Differentiation\*

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Anti-idiotypic antibodies have been used to demonstrate unique idiotypic determinants on the cell surface of affected clones of peripheral blood lymphocytes from patients with macroglobulinemia (1), multiple myeloma (2-6), benign monoclonal gammopathy (7), and chronic lymphocytic leukemia associated with monoclonal gammopathy (8-11). Although multiple myeloma is classically considered a malignancy of bone marrow plasma cells, the above studies have suggested that B-lymphocyte clonal ancestors of the malignant plasma cells may also be expanded. Reports of the existence of both T and B lymphocytes bearing identical idiotypic (Id)<sup>1</sup> determinants in two patients with IgG<sub>λ</sub> myelomas (5, 6) suggest that the target cell for oncogenic transformation could be a precursor cell for both B- and T-cell clones.

It is now evident from ontogenetic studies in mammals (man, mouse, and rabbit) that the appearance of B lymphocytes bearing surface IgM in fetal liver is preceded by the development of a population of lymphoid cells, which contain small amounts of cytoplasmic IgM components but lack surface immunoglobulins detectable by immunofluorescent techniques (12-14). These pre-B cells are presumed to be direct precursors of B lymphocytes (15-17). After lympho-hemopoiesis ceases in fetal liver, large, rapidly dividing pre-B cells are found exclusively in the bone marrow (12-14), which is now recognized as the adult source of newly generated B lymphocytes in mammals (18, 19), analogous to the avian bursa of Fabricius (20). It has recently been found that in  $\cong 20\%$  of individuals with childhood acute lymphocytic leukemia,

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<sup>1</sup> Abbreviations used in this paper: BSA, bovine serum albumin; Cohn Fx II, pooled normal human gammaglobulin; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Id, idiotype; Ind Id, individually-specific idiotype; MR Id, myeloma-related idiotype; PBS, phosphate-buffered saline; RITC, rhodamine isothiocyanate; sIg, surface immunoglobulin; SRBC, sheep erythrocytes; V<sub>H</sub>, variable portion of immunoglobulin heavy chain; V<sub>L</sub>, variable portion of immunoglobulin light chain.

the malignant cells have the pre-B cell phenotype (21). Possible involvement of pre-B cells in other malignancies of B-cell lineage has not been investigated.

In this study, we have used antibodies against idiotypic determinants of myeloma proteins to analyze the developmental history of multiple myelomas by immunofluorescent techniques. The homologous idiotypic determinants were found exclusively in plasma cells containing IgA in two patients with IgA myeloma, whereas on the surface of circulating B lymphocytes the same determinants were associated with different immunoglobulin classes. An expanded population of IgM-containing pre-B cells expressing the homologous idio type was found in each patient, whereas T lymphocytes were not found to express the idiotypic determinants. These findings point to oncogenic transformation very early in the life history of an affected B-cell clone in multiple myeloma.

### Materials and Methods

*Isolation and Characterization of Monoclonal Immunoglobulins.* Sera were collected from two patients with IgA myeloma, IgA<sub>x</sub> (001) and IgA<sub>x</sub> (003), before therapy. Crude globulins, isolated from serum by precipitation with sodium sulfate, were dialyzed against 0.01 M Tris-HCl buffer, pH 8.0 and applied to a column of DEAE cellulose (DE 52, Whatman Inc., Clifton, N. J.) equilibrated with the same buffer. Fractions containing myeloma IgA were eluted with a linear gradient to 0.5 M NaCl-0.01 M Tris-HCl buffer pH 8.0, and chromatographed on a column of Sephadex G-200 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) equilibrated with borate-saline buffer, pH 8.3 (ionic strength = 0.1). The exclusion peak containing polymeric IgA was collected and concentrated. Residual IgG was removed by passage over an affinity column bearing rabbit antibodies to human  $\gamma$ -chains. The purity of IgA paraproteins was tested by immunoelectrophoresis and double diffusion in agar with antisera to normal human serum proteins, IgG, IgA, and IgM. Heavy and light chains were prepared from purified myeloma immunoglobulins by reduction and alkylation followed by separation on Sephadex G-200 in 5 M guanidine-HCl, pH 7.2.

Amino-terminal sequencing to 20 residues was used to ascertain V<sub>H</sub> and V<sub>L</sub> subgroups (22). IgA protein 001 had a V<sub>H1</sub> heavy chain and V<sub>L1</sub> light chain; IgA 003 was V<sub>HIII</sub> and V<sub>LIV</sub>. IgA myeloma proteins BLK and STC were selected as matches for 001 and 003 with respect to V<sub>H</sub> and V<sub>L</sub> subgroups as well as C<sub>H</sub> and C<sub>L</sub> sequence.

*Immunization.* 1 mg of purified myeloma protein in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) was injected subcutaneously into rabbits or goats. Booster injections, with 1 mg of myeloma protein in incomplete Freund's adjuvant, were given two to four times at 10-d intervals from day 14 after primary immunization.

*Preparation of Anti-idiotypic Antibodies.* Solid immunoadsorbent columns were prepared by covalent attachment of normal serum proteins or myeloma proteins to Sepharose 4B activated with cyanogen bromide (23). Antisera were absorbed sequentially to remove unwanted specificities; each absorption was repeated until the quantity of absorbed protein eluted from the column by 0.05 M glycine-HCl buffer, pH 2.8, was less than 0.5 mg. The absorbed antisera were then passed over a column containing the homologous myeloma protein and the bound anti-Id antibodies eluted. A representative absorption sequence is shown in Table I in which the relative yield of anti-Id is indicated.

*Direct Hemagglutination Assays.* Human erythrocytes (type 0, Rh +) were coated with myeloma proteins or normal human immunoglobulins (Cohn Fx II) or anti-Id antibodies by the chromic chloride method (24). Serial dilutions of purified goat or rabbit anti-Id or goat anti-human F(ab')<sub>2</sub> antibodies in phosphate-buffer saline (PBS) containing 5% bovine serum albumin (BSA) were prepared in plastic microwells (Cooke Laboratory Products, Alexandria, Va.) to which immunoglobulin-coated erythrocytes were added. As a reciprocal test, serial dilutions of myeloma proteins or Cohn Fx II were mixed with anti-Id coated erythrocytes.

*Solid-phase Inhibition Radioimmunoassay.* Purified myeloma proteins IgA<sub>x</sub> (001) and IgA<sub>x</sub> (003), were labeled with carrier-free <sup>125</sup>I (New England Nuclear, Boston, Mass.) by the chloramine T method (25). Polystyrene tubes (Beckman Instruments, Inc., Fullerton, Calif.) were coated for

4 h at room temperature with 0.5  $\mu\text{g}/\text{ml}$  of purified anti-Id. These quantities were based on preliminary estimates of amounts of antibody which would bind 30% of the  $^{125}\text{I}$ -labeled homologous myelomas. In some experiments, polystyrene tubes were coated with saturating amounts of anti-Id antibodies. After washing out excess antibody with PBS and coating free sites on tubes with 0.1% BSA in PBS, several concentrations of different inhibitor proteins were added to the antibody-coated tubes in triplicate and incubated for 1 h at 37°C. 100  $\mu\text{l}$  of  $^{125}\text{I}$ -labeled homologous myeloma protein ( $4 \times 10^5$  cpm/ml) was then added to each tube for an additional 20 h at 37°C. Unbound radioactivity was washed out with PBS and the tubes were counted in a gamma counter.

**Conjugation.** F(ab')<sub>2</sub> fragments of rabbit anti-Id antibodies were prepared by pepsin digestion (26), separated from residual intact molecules by chromatography on Sephadex G-150, and then absorbed with Staphylococcal Protein A (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) coupled to Sepharose 4B. Purified F(ab')<sub>2</sub> fragments of rabbit anti-Id, or intact goat anti-Id, were conjugated with fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC) as previously described (27).

**Immunofluorescence Analysis of Cells.** Peripheral blood lymphocytes, isolated by Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.)-Hypaque (Winthrop Laboratories, N. Y.) gradient centrifugation and bone marrow cells, depleted of mature erythrocytes by lysis with a hypotonic solution of ammonium chloride, were washed with PBS supplemented with 10% fetal calf serum (FCS) (Gibco Diagnostics, Gibco Invenex Div., Chagrin Falls, Ohio), resuspended in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) containing 20% FCS and incubated in plastic dishes for 45 min at 37°C to remove adherent cells and cytophilic immunoglobulin. To characterize the cells bearing idiotypic determinants, previously described methods for two-color immunofluorescence were used for double staining of cell-surface immunoglobulins, simultaneous staining of cell-surface and cytoplasmic immunoglobulins and sequential staining of two cytoplasmic immunoglobulin determinants with FITC- or RITC-labeled antibodies (13). The antibodies used for counterstaining with fluorochrome-labeled anti-Id antibodies included purified goat antibodies with the following specificities for human immunoglobulin determinants: anti- $\mu$ , anti- $\delta$ , anti- $\gamma$ , anti- $\alpha$ , anti-F(ab')<sub>2</sub>, anti- $\kappa$  and anti- $\lambda$  prepared as described elsewhere (13).

**T- and B-Cell Separation.** T cells were isolated from peripheral blood by rosette formation with neuraminidase-treated sheep erythrocytes (SRBC) and density gradient separation as described (28). The contamination of the T-cell-enriched fraction with surface immunoglobulin-positive cells was <1%. Fluoresceinated rabbit antiserum for the detection of human T cells was kindly supplied by Dr. Charles M. Balch et al. (29).

## Results

**Specificity of Purified Anti-idiotypic Antibodies.** The specificity of purified anti-Id antibodies was studied by two sensitive assays, direct hemagglutination and inhibition radioimmunoassay. Less than 0.1 ng/ml of homologous myeloma protein agglutinated erythrocytes coated with purified anti-Id, more than  $10^7$  times greater concentrations of pooled normal immunoglobulins (Cohn Fx II protein), or of myeloma proteins matched for heavy-chain class, light-chain type, and V<sub>H</sub> and V<sub>L</sub> subgroups, were required to achieve hemagglutination (Table II). Comparable results were obtained when the reverse assay, in which anti-Id antibodies were used to agglutinate antigen-coated erythrocytes.

Radioimmunoassays indicated that both anti-Id (001) and anti-Id (003) recognize determinants of both light and heavy chains of the homologous myeloma proteins (Fig. 1). However, the inhibitory capacity (50% inhibition) of the isolated chains was at least 100-fold less than that of the intact myeloma proteins. Neither Cohn Fx II protein nor other IgA myeloma proteins inhibited binding of anti-Id (001) at the highest concentrations tested, whereas for anti-Id (003),  $5 \times 10^6$  ng/ml of Cohn Fx II protein gave 25% of the inhibition that was given by 4 ng/ml of IgA $_{\lambda}$  (003). Even

TABLE I  
Purification of Goat Antibodies to Idiotypic Determinants of IgA Myeloma Protein (001)

Immunoabsorbent columns	Percentage of total bound antibodies*
IgG <sub>κ</sub> (×2)‡	38.4
IgM <sub>κ</sub>	3.3
IgG <sub>λ</sub>	0.8
Normal IgG (×3)	6.4
Normal F(ab') <sub>2</sub>	1.0
IgA <sub>κ</sub> (FH) (×2)	26.0
IgA <sub>λ</sub> (BG) (×2)	1.0
IgA <sub>λ</sub> (JP)	0.9
Cohn Fx II protein	0.1
IgA <sub>κ</sub> (001)	22.1§

\* Quantity of protein eluted from each column divided by the total proteins eluted from all columns times 100.

‡ Numbers in parentheses indicate multiple column absorptions.

§ From an initial aliquot of 45 ml of goat anti-IgA<sub>κ</sub> (001) antiserum, 33.7 mg of anti-Id (001) antibodies were obtained. In different sera, anti-Id antibodies represented 10–20% of the total antibodies reactive with immunoglobulin determinants.

TABLE II  
Specificity Test of Purified Anti-idiotypic Antibodies

Erythrocyte-coating antibodies	Hemagglutinating protein	Minimum amount for hemagglutination
		ng/ml
Anti-Id (001)	IgA <sub>κ</sub> (001)*	<0.03
Anti-Id (001)	IgA <sub>κ</sub> (BLK)*	1,000,000
Anti-Id (001)	Cohn Fx II	10,000,000
Anti-Id (003)	IgA <sub>λ</sub> (003)‡	<0.1
Anti-Id (003)	IgA <sub>λ</sub> (STC)‡	>1,000,000
Anti-Id (003)	Cohn Fx II	>10,000,000

\* V<sub>H</sub> and V<sub>L</sub> subgroups of these two myeloma proteins were the same based on the analysis of N-terminal 20 amino acids; V<sub>H1</sub> and V<sub>κ1</sub>.

‡ V<sub>H11</sub> and V<sub>λ1V</sub>.

when tubes were coated with saturating amounts of anti-Id antibodies, IgA myeloma proteins matched for light-chain type and V<sub>H</sub> and V<sub>L</sub> subgroups did not inhibit binding at the highest concentration tested ( $3 \times 10^4$  ng/ml).

*Expression of Idiotypic Determinants on Lymphocytes from Normal Donors.* Given their apparent specificity for the immunizing myelomas in the hemagglutination assays and radioimmunoassay, fluorochrome-labeled anti-Id antibodies stained a surprisingly high proportion of B lymphocytes from normal donors (Table III). Goat anti-Id (001) stained  $0.8 \pm 0.3\%$  of sIg<sup>+</sup> cells, whereas F(ab')<sub>2</sub> fragments of rabbit anti-Id (003) stained  $0.5 \pm 0.3\%$ . The staining of normal lymphocytes by F(ab')<sub>2</sub> fragments of anti-Id antibodies was completely inhibited in the presence of a 16-fold excess of the homologous myeloma protein, but was not inhibited by myeloma proteins of the same heavy-chain isotype, light-chain class and V<sub>H</sub> and V<sub>L</sub> subgroup. Idiotypic determinants were found in association with all classes of cell surface immunoglobulins but

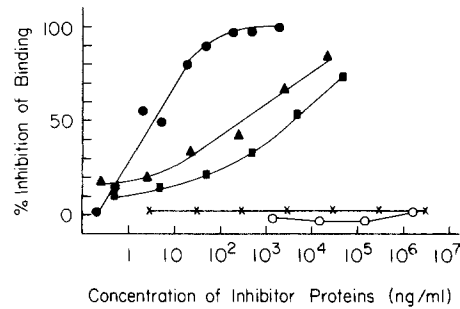


FIG. 1. Effect of various inhibitor proteins on the binding of  $^{125}\text{I}$ -IgA $\kappa$  (001) by anti-Id (001). Each point is the average of triplicate assays. Polystyrene tubes were coated with 0.5  $\mu\text{g}/\text{ml}$  of anti-Id (001), which gave 30% of maximum binding of  $^{125}\text{I}$ -IgA $\kappa$  (001). IgA $\kappa$  (001) (●),  $\alpha$ -chain (001) (▲),  $\kappa$ -chain (001) (■), Cohn Fx II proteins (×), and unrelated IgA myeloma protein (○) were used as inhibitors.

TABLE III

*Expression of Myeloma-related Idiotypes on Peripheral B Lymphocytes from Six Normal Subjects\**

A. Frequency of Id <sup>+</sup> cells among B lymphocytes bearing different isotypes					
Idiotype	$\frac{\text{Id}}{\text{Ig}^+}$	$\frac{\text{Id}}{\mu^+}$	$\frac{\text{Id}}{\delta^+}$	$\frac{\text{Id}}{\gamma^+}$	$\frac{\text{Id}}{\alpha^+}$
001	0.8 $\pm$ 0.3	1.2 $\pm$ 0.4	1.3 $\pm$ 0.4	1.3 $\pm$ 0.9	0.6 $\pm$ 0.3
003	0.5 $\pm$ 0.3	0.6 $\pm$ 0.3	0.6 $\pm$ 0.5	1.2 $\pm$ 0.4	0.4 $\pm$ 0.1
B. Distribution of isotype among Id <sup>+</sup> B lymphocytes					
Idiotype	$\frac{\mu^+\text{Id}^+}{\text{Id}^+}$	$\frac{\delta^+\text{Id}^+}{\text{Id}^+}$	$\frac{\gamma^+\text{Id}^+}{\text{Id}^+}$	$\frac{\alpha^+\text{Id}^+}{\text{Id}^+}$	
001	73 $\pm$ 18	74 $\pm$ 19	55 $\pm$ 38	8 $\pm$ 4	
003	73 $\pm$ 31	67 $\pm$ 21	71 $\pm$ 27	10 $\pm$ 1	
C. Distribution of light chain type among Id <sup>+</sup> B lymphocytes					
Idiotype	$\frac{\kappa^+\text{Id}^+}{\text{Id}^+}$	$\frac{\lambda^+\text{Id}^+}{\text{Id}^+}$			
001	75 $\pm$ 21	25 $\pm$ 21			
003	1 $\pm$ 3	99 $\pm$ 3			

\* Frequencies of B lymphocytes were as follows: 16.3  $\pm$  3.9% Ig<sup>+</sup>-B cells, 10.2  $\pm$  4.3%  $\mu^+$ -B cells, 10.1  $\pm$  3.9%  $\delta^+$ -B cells, 5.8  $\pm$  2.1%  $\gamma^+$ -B cells, and 2.1  $\pm$  0.9%  $\alpha^+$ -B cells. All values are expressed as mean  $\pm$  SD.

more frequently on lymphocytes expressing  $\mu$ -,  $\delta$ -, or  $\gamma$ -heavy chains than those expressing  $\alpha$ -chain. The combined frequencies of  $\mu^+$ ,  $\delta^+$ , and  $\gamma^+$ -cells among Id<sup>+</sup> cells, determined independently, added to >200%. This is consistent with the co-expression of  $\mu$ - and  $\delta$ -chains by most B lymphocytes, and the observation that approximately one-third of  $\gamma^+$ -B-lymphocytes in adult blood also express  $\mu$ - and/or  $\delta$ -chains (13). In these experiments, cells were allowed to cap after exposure to anti-Id and before staining for Ig isotype under conditions which prevented capping. We never observed independent distribution of the two fluorochromes, indicating that anti-Id and anti-isotype antibodies were reacting with the same molecules.

Anti-Id (001), raised to an IgA $\kappa$  myeloma, recognized sIg of  $\kappa$ - and  $\lambda$ - light-chain type in a ratio of  $\approx 3:1$  in seven of eight different samples of peripheral lymphocytes. With anti-Id (003), raised to an IgA $\lambda$  myeloma, only a single Id<sup>+</sup> $\kappa^+$  cell was found in

the same eight samples. These data may relate to differences in the specificity of the two antibody preparations for idiotypic determinants of  $\alpha$ - and light chains, respectively. Anti-Id (001) had a relatively greater affinity for homologous heavy chain than for light chain (Fig. 1), whereas the reverse was true for anti-Id (003) (data not shown). The data obtained with anti-Id (001) is consistent with the possibility that the  $V_H$  determinants recognized by these antibodies may be expressed in conjunction with several different light chain V-regions and vice-versa. In experiments in which cells were stained with both preparations of anti-Id antibodies, no doubly stained cells from either normal individuals or the patients were observed.

The anti-Id antibodies reacted with normal plasma cells of all Ig classes in the same or slightly higher frequencies than those obtained for B lymphocytes. Plasma cells expressing both idiotypic specificities were not observed, and anti-Id antibodies did not stain representative myeloma cells of all major immunoglobulin classes (IgM, IgD, IgG, and IgA) and types ( $\kappa$  and  $\lambda$ ). In cultures of peripheral blood lymphocytes stimulated with pokeweed mitogen, the frequencies of plasma cells containing the idiotypic determinants correlated closely with the frequency of Id<sup>+</sup> B lymphocytes in the preculture population (data not shown).

*Isolation of Subpopulations of Anti-idiotypic Antibodies.* The above observations strongly suggested that these two preparations of anti-Id were not detecting two unique clones of B cells, but rather two distinct families of cells expressing myeloma-related idiotypic determinants. We therefore attempted to refine the specificity of these reagents by repeated absorption of 2 mg of anti-Id antibody over a column loaded with 220 mg of normal gammaglobulin (Cohn Fx II protein) (30). The relative capacity to agglutinate erythrocytes coated with the homologous myeloma protein as opposed to erythrocytes heavily coated with Cohn Fx II protein was not altered by five such absorptions (data not shown). However, the frequency of B lymphocytes reactive with these absorbed antibody preparations was dramatically reduced. The further absorbed anti-Id (001) stained <0.03% of B lymphocytes from normal donors, as compared to  $0.8 \pm 0.3\%$  for the original anti-Id (001) preparation. Absorbed and original anti-Id (003) preparations stained  $\leq 0.03\%$  and  $0.5 \pm 0.3\%$  of normal sIg<sup>+</sup> B lymphocytes, respectively. In view of these results, the original preparations of anti-Ids will be referred to as anti-myeloma related (MR) Id and the further absorbed anti-Ids with finer specificity as anti-individual (Ind) Id.

*Exclusive Expression of Idiotype by B Cells in Patients with Multiple Myeloma.* As shown in Table IV, Id<sup>+</sup> cells could not be detected in the T-cell-enriched fraction of lymphocytes from either patient, whereas from 5 to 8% of sIg<sup>+</sup> cells in B-cell-enriched fractions were also stained for idiotype.

*Relationship of Idiotype to Isotype Expression by B Lymphocytes and Plasma Cells of Myeloma Patients.* Preparations of circulating lymphocytes and bone marrow cells from the myeloma patients were simultaneously stained for surface idiotype and isotype or for cytoplasmic idiotype and isotype, respectively. In these experiments both anti-MR Id and anti-Ind Id were used. Results are shown in Table V. The great majority of plasma cells in bone marrow from both patients were IgA-positive. Of these, >94% stained brightly with the corresponding anti-Id. The frequencies of Id<sup>+</sup>IgA<sup>+</sup> plasma cells detected were identical whether idiotypic determinants were detected by anti-MR Id or anti-Ind Id, and no plasma cells containing intracytoplasmic IgM or IgG co-stained for idiotypic determinants.

TABLE IV  
*Expression of Myeloma-related Idiotypes on Peripheral B- and T-Lymphocyte Populations in Myeloma Patients*

Surface marker	Patient 001	Patient 003
B-cell fraction:		
sId <sup>-</sup> sIg <sup>+</sup>	19.2*	27.5
sId <sup>+</sup> sIg <sup>+</sup>	1.0	2.5
sId <sup>+</sup> sIg <sup>-</sup>	<0.1	<0.1
T-cell fraction:		
sId <sup>-</sup> anti-T <sup>+</sup>	61.3	68.2
sId <sup>+</sup> anti-T <sup>+</sup>	<0.05	<0.05
sId <sup>-</sup> ER <sup>+</sup> ‡	ND§	66.6
sId <sup>+</sup> ER <sup>+</sup> ‡	ND	<0.1

\* Values are expressed as the percentage of lymphocytes with the indicated phenotype among each fraction.

‡ Cells were first stained for surface idiotype, then rosettes were formed with neuraminidase-treated SRBC. Evaluation was done on wet-mount preparations.

§ Not done.

TABLE V  
*Expression of Idiotype Determinants in Plasma Cells and B Lymphocytes from Myeloma Patients*

Donor*		Anti-Id‡	Percent of Id <sup>+</sup> cells among:							
			Marrow plasma cells			Peripheral B lymphocytes				
			IgM	IgG	IgA	sIgM	sIgD	sIgG	sIgA	sIg
001	Exp. 1	Anti-MR Id	0	0	99	18.6	8.7	7.8	13.7	11.2
		Anti-Ind Id	0	0	99	<0.4	<0.4	0.2	3.2	2.2
	Exp. 2	Anti-MR Id	ND§	ND	ND	14.4	27.4	24.3	38.1	22.4
		Anti-Ind Id	ND	ND	ND	1.6	2.1	5.4	11.1	7.1
003		Anti-MR Id	0	0	94	1.3	1.5	3.5	9.6	3.1
		Anti-Ind Id	0	0	94	0.2	0.1	0.1	1.0	1.4

\* Frequencies of B lymphocytes among peripheral blood lymphocytes for patient 001 were 1.3 and 1.6% Ig<sup>+</sup> B cells, 0.4 and 0.3%  $\mu^+$  B cells, 0.5 and 0.6%  $\delta^+$  B cells, 0.5 and 0.6%  $\gamma^+$  B cells, and 0.4 and 0.4%  $\alpha^+$  B cells, and for patient 003, 9.6% Ig<sup>+</sup> B cells, 7.7%  $\mu^+$  B cells, 6.1%  $\delta^+$  B cells, 0.8%  $\gamma^+$  B cells, and 0.6%  $\alpha^+$  B cells.

‡ Anti-Ind Id (001) stains <0.03% of normal B lymphocytes, whereas anti-Ind Id (003) stains  $\leq$ 0.03%. Anti-MR Id and anti-Ind Id antibodies were used at the same concentration (0.5 mg/ml).

§ Not done.

Different results were obtained with regard to Id<sup>+</sup> B lymphocytes. Among sIg<sup>+</sup> circulating lymphocytes from patient 001, 11 and 22% (in two experiments) also had idiotypic determinants detected by anti-MR Id (001). Idiotypic determinants were detected on cells bearing IgM, IgD, IgG, and IgA. When stained with anti-Ind Id (001), the frequency of sIg<sup>+</sup> cells costained for idiotype was reduced three- to fivefold, and positive cells were largely sIgA<sup>+</sup> lymphocytes. In one of the two experiments, no lymphocytes bearing IgM or IgD were Id<sup>+</sup>. Among sIg<sup>+</sup> cells from patient 003, 3.1% costained with anti-MR Id (003) and 1.4% with anti-Ind Id (003). Using either anti-Id reagent, Id<sup>+</sup> cells expressing each of the major isotypes were detected, although the majority of Id<sup>+</sup> cells were sIgA<sup>+</sup>.

In summary, these data indicate that the frequency of Id<sup>+</sup> cells among B lympho-

cytes from myeloma patients detected by anti-Ind Id is 50–250 times higher than among normal B lymphocytes. In both patients, idiotypic determinants were present on lymphocytes bearing surface Ig molecules of different heavy-chain isotypes, but the predominant class of Id<sup>+</sup> B lymphocytes detected by anti-Ind Id consisted of cells expressing the same isotype as the myeloma plasma cells.

The anti-Ind Id antibodies apparently stain all cells belonging to their respective myelomatous clones. The anti-MR Id antibodies stain the same cells, and an additional population of B lymphocytes producing immunoglobulins having related V<sub>H</sub> and/or V<sub>L</sub> determinants. In patient 001, for example, the difference in frequencies of Id<sup>+</sup> cells detected by anti-MR Id and anti-Ind Id was 9–15%. The frequency of B lymphocytes bearing myeloma-related idiotypes is therefore >10 times as high in patient 001 than in a group of normal donors (0.8% of sIg<sup>+</sup> cells). Similarly, the relative frequency of B lymphocytes bearing myeloma-related idiotypes in patient 003 is several fold higher than among B lymphocytes from normal donors.

*Presence of Idiotypic Determinants in Pre-B Cells from Myeloma Patients.* Pre-B cells can be distinguished from B lymphocytes by the lack of surface IgM, and from plasmablasts and plasma cells by their lymphoid morphology, relatively weak cytoplasmic immunofluorescence, and lack of sIgM (which is present on the majority of IgM-containing plasma cells). Two staining techniques were used to search for pre-B cells containing idiotypic determinants. In the first, viable nucleated bone marrow cells were stained with FITC anti- $\mu$  or anti-F(ab')<sub>2</sub>, fixed, and then stained with RITC anti-Id or RITC anti- $\mu$ . From such preparations the frequencies of sIgM<sup>+</sup> B lymphocytes and of sIg<sup>-</sup>cIgM<sup>+</sup> pre-B cells were determined. Id<sup>+</sup> pre-B cells were defined by their lymphoid morphology, lack of surface Ig determinants, and weak cytoplasmic fluorescence with anti-Id. This method excludes the possibility of scoring sIgM<sup>+</sup>sId<sup>+</sup> B lymphocytes as pre-B cells, but might falsely include a population of plasmablasts containing small amounts of IgA of the myeloma idiotype. Control experiments were done to estimate the frequency of such cells. It was first determined that virtually all (>99%) IgA-containing cells in the marrow preparations from either patient had detectable surface IgA. In preparations stained with anti-F(ab')<sub>2</sub> to detect surface Ig and anti- $\alpha$  to detect cytoplasmic IgA, the frequency of sIg<sup>-</sup>cIgA<sup>+</sup> cells was  $\cong$ 1 in 20,000 nucleated cells. This was 10-fold less than the sIg<sup>-</sup>cId<sup>+</sup> or of sIgM<sup>-</sup>cId<sup>+</sup> cells with pre-B-cell morphology.

In the second method, fixed preparations of marrow cells were stained sequentially with RITC anti- $\mu$  and FITC anti-Id antibodies; pre-B cells were defined as lymphoid cells costaining for IgM and idiotypic determinants. With this method, some sIgM<sup>+</sup>cId<sup>+</sup> cells might be incorrectly scored as pre-B cells, but cIgA<sup>+</sup>cId<sup>+</sup> cells would be excluded. In one experiment, bone marrow cells were incubated on a plastic dish coated with anti-Ig. The frequency of sIgM<sup>+</sup> cells in the nonadherent population was reduced fourfold (to 0.16%), whereas the frequency of cIgM<sup>+</sup>cId<sup>+</sup> cells was increased 11-fold (to 0.23%). Thus, we believe most of the cells scored in this manner are indeed sIg<sup>-</sup> pre-B cells.

To further limit subjectivity, slides were independently examined by four different observers. Finally, two different counting methods were used for slides stained for cIgM and cId. The frequency of cIgM<sup>+</sup>cId<sup>+</sup> cells as a function of nucleated cells was determined first. A second observer then scanned the entire slide to determine the frequency of cId<sup>+</sup> cells among total cIgM<sup>+</sup> cells.



TABLE VI  
*Expression of Idiotype By Pre-B Cells in Bone Marrow \**

Donor	Months of treatment‡	Reagents and Staining Procedure			
		Anti- $\mu$ , fix, anti-MR Id, or anti- $\mu$		Fix, anti- $\mu$ , and anti-MR Id	Fix, anti- $\mu$ , and anti-Ind Id
		Date	s $\mu$ <sup>-</sup> c $\mu$ <sup>+</sup> pre-B cell	s $\mu$ <sup>-</sup> cId <sup>+</sup> pre-B cell	c $\mu$ <sup>+</sup> cId <sup>+</sup> pre-B cell
001	0	0.13	0.06 (46)§	0.04 (31)	0.05 (39)
	3	0.30	0.17 (57)	0.10 (33)	ND
	18	0.11	ND	<0.01 (<9)	0.006 (6)
Normal control		0.87	<0.003 (<0.3)	ND	ND
003	6	0.32	ND	0.04 (13)	0.024 (7.5)
	14	0.32	ND	0.02 (6)	ND
	20	0.04	0.03 (75)	0.006 (15)	0.004 (10)
Normal control		0.87	≤0.005 (≤0.6)	ND	ND

\* Results are expressed as the percentage of cells with the indicated phenotype among nucleated bone marrow cells.

‡ Patient 001 was treated with prednisone and chlorambucil, and patient 003 with prednisone and melphalan.

§ Numbers in parentheses indicate the percentage of Id<sup>+</sup> pre-B cells among total pre-B cells.

|| Not done.

Data from these experiments are presented in Table VI. Pre-B cells were detected by both staining methods. Frequency estimates were higher when sIgM<sup>-</sup>cId<sup>+</sup> cells were determined than when cIgM<sup>+</sup>cId<sup>+</sup> cells were scored as pre-B cells. The results shown were made by a single observer and are conservative; independently determined frequencies were either the same or higher by not more than twofold. Also shown is a comparison of frequencies obtained using anti-MR Id and anti-Ind Id. Pre-B cells were detected by either reagent in approximately the same numbers.

In preparations of normal marrow, no pre-B cells (sIgM<sup>-</sup>cId<sup>+</sup>) stained for the idiotype among 450 cells examined with either the anti-MR Id (001) or anti-Ind Id (001). A single positive cell was observed with MR anti-Id (003). In other experiments using anti-MR Id to stain normal or fetal bone marrow, the frequency of Id<sup>+</sup> pre-B cells has never exceeded 1%, and most specimens have lacked positive cells (31). Our results, therefore, indicate that the frequency of pre-B cells bearing the homologous idiotype is significantly increased in myeloma patients. The proportionate increase of Id<sup>+</sup> pre-B cells was greater than that for circulating Id<sup>+</sup> B lymphocytes determined simultaneously.

### Discussion

In the course of these studies on the extent of clonal involvement in IgA myelomas in two individuals, we prepared two distinct sets of anti-idiotype antibodies for each myeloma protein. Purified antibodies to the IgA<sub>k</sub> and IgA<sub>λ</sub> myeloma proteins obtained by the initial series of absorptions using affinity columns did not react with myeloma proteins matched with respect to light-chain type, heavy-chain class, and V<sub>H</sub> and V<sub>L</sub> subgroups. The specificity of these reagents was largely directed towards conformational determinants of combined heavy and light chains, because the isolated chains

had 100- to 5,000-fold less inhibitory capacity than the intact proteins in an inhibition radioimmunoassay. Using either this assay or a direct hemagglutination assay, the frequency of Id<sup>+</sup> molecules among normal serum immunoglobulins was estimated to be no greater than 1 in 10<sup>6</sup>, a figure in agreement with that reported by Kunkel for individual myeloma-specific antibodies (32). However, fluorochrome conjugates of these anti-Id reagents stained 0.5–0.8% of sIg<sup>+</sup> cells from normal individuals, frequencies several orders of magnitude higher than might be expected for individual clones. Some investigators using immunofluorescence techniques have reported similar or higher frequencies of sId<sup>+</sup> lymphocytes in normal individuals (2, 4, 33, 34), whereas others have detected none (5, 8, 9, 35). Therefore, the purified anti-Id antibodies were passed repeatedly over affinity columns containing large amounts of pooled human gammaglobulin. The frequency of sId<sup>+</sup> cells was thereby reduced to <0.03% of normal B lymphocytes. This procedure provided a derivative set of anti-Id antibodies having narrow specificity; the paired sets of reagents were called anti-MR Id and anti-Ind Id for reasons outlined below.

In support of the interpretation that the initial anti-Id reagents were specific for myeloma-related idiotypic determinants are the following observations: (a) staining of normal lymphocytes by anti-MR Id could be completely inhibited by the homologous myeloma, but was not inhibited by myelomas of the same heavy-chain isotype, light-chain class and V<sub>H</sub> and V<sub>L</sub> subgroups; (b) anti-MR Id antibodies did not stain plasma cells from other patients with myeloma ( $\mu$ -,  $\delta$ -,  $\gamma$ -,  $\alpha$  heavy chains,  $\kappa$ - and  $\lambda$ -light chains); (c) anti-Id antibodies costained lymphocytes bearing  $\mu$ -,  $\delta$ -,  $\gamma$ -, and  $\alpha$ -isotypic determinants, and, in the case of anti-Id (IgA $\kappa$ ), lymphocytes bearing both  $\kappa$ - and  $\lambda$ -light chains; (d) the frequency of sId<sup>+</sup> lymphocytes among B lymphocytes from normal donors was similar to the frequency of cId<sup>+</sup> plasma cells among total plasma cells in cultures stimulated with pokeweed mitogen, and (e) idiotypic determinants were detected in cells containing IgM, IgG, and IgA immunoglobulins. The sum of our observations on reagent specificity is most consistent with the following interpretation: the anti-MR Id antibodies detect a family of clones expressing antibodies with structurally-related Id determinants whereas the derivative anti-Ind Id antibodies react with a more restricted set of idiotypes within the same family.

Neither set of anti-Id preparations reacted with T lymphocytes from the two patients or from normals. The differences between this observation and that of several laboratories supporting the existence of idiotypic determinants on T lymphocytes (5, 6, 33, 36–38), might be based upon the properties of different anti-Id preparations or upon differences in the patients. We have also failed to find Id<sup>+</sup> T cells in two additional myeloma patients with IgM and IgD paraproteins.

Idiotypic determinants expressed by plasma cells from our IgA-myeloma patients were restricted to IgA-producing cells, and were detected equally by anti-MR Id and anti-Ind Id. This was not true for B lymphocytes. First, lymphocytes bearing cell surface IgM, IgD, and IgG as well as IgA were costained for Id. Secondly, anti-MR Ids stained substantially higher proportions of B cells than did anti-Ind Ids. This suggests the existence of two expanded populations of B lymphocytes in our patients. One, detected by the anti-Ind Id, includes primarily cells bearing the unique myeloma idio type; a high proportion of these cells express the same isotype (IgA) as the myeloma. The second population, represented by the difference in frequencies of Id<sup>+</sup> cells detected by anti-MR Id and anti-Ind Id, consists of cells expressing related but

not identical idiotypes which are expressed predominantly on sIgM<sup>+</sup> and sIgD<sup>+</sup> lymphocytes. This second population appeared to be 2–10 times as frequent among B lymphocytes from the patients than among normal B lymphocytes.

The mechanism responsible for the apparent expansion of B-cell clones expressing idiotypes related to the myelomatous clone is unknown. One interesting possibility is suggested by the network hypothesis, which proposes that immune responses are regulated, in part, by formation of auto anti-idiotypic antibodies (39). Treatment of mice with heterologous anti-idiotypic antibodies results in an expansion of clones of B cells expressing that idio type similar to the expansion induced by specific antigen (40). Whether antigen or anti-idiotypic antibodies are used for priming, only about one-half of the expanded clones of Id<sup>+</sup> cells produce antibodies reactive with the appropriate antigen (40, 41). The Id<sup>+</sup> clones reactive with the relevant antigen in these systems might be considered analogous to the unique myeloma clone in our patients, whereas the clones with related idiotypes, but different combining sites, could be similar to the clones detected by anti-MR Id.

The correspondence of frequencies of plasma cells detected by anti-MR Id and anti-Ind Id in our patients and the failure to find Id<sup>+</sup> plasma cells of classes other than IgA, suggests that the population of B lymphocytes detected by anti-MR Id was not induced to undergo terminal differentiation.

Observations in this and earlier studies (2–7) that the myeloma idio type is expressed by a high proportion of B lymphocytes in patients imply that the oncogenic event occurs at an earlier stage of B-cell differentiation than the plasma cell. The concept is supported by our finding that among the Id<sup>+</sup> B lymphocytes were populations expressing sIgM, sIgG, and sIgD, as well as sIgA. Because all B-cell clones appear to go through a developmental phase during which all cells express IgM only before giving rise to B cells expressing the other immunoglobulin classes (13, 42, 43), the oncogenic event in both patients must have occurred at least as early as the immature sIgM<sup>+</sup> B lymphocyte.

Consistent with this interpretation, we observed what appeared to be Id<sup>+</sup> pre-B cells of the myeloma clones. In contrast to our observations on B lymphocytes, frequencies of pre-B cells determined with anti-MR Id and anti-Ind Id preparations were virtually identical. This observation suggests that the Id<sup>+</sup> pre-B cells in the patients belong to the unique myeloma clone rather than idiotypically related clones. It follows that the expanded population of B lymphocytes expressing myeloma-related idiotypes in the myeloma patients is probably not derived from an expanded population of pre-B cells. This idea is consistent with the speculation that auto-anti-idiotypic antibodies or T cells with anti-idiotypic receptors may play a role in generating the increased proportion of MR Id<sup>+</sup> B lymphocytes. Neither anti-Id antibodies nor T cells would be expected to influence pre-B cells of myeloma related clones because pre-B cells lack easily detectable sIg (44), and have been shown not to be numerically or functionally influenced by exposure to anti-IgM antibodies under conditions in which development of B lymphocytes and plasma cells is almost completely blocked (17).

The demonstration of idio typic determinants in pre-B cells has other important implications. We have previously shown that pre-B cells from rabbits may express light chains as well as  $\mu$ -chains, and that pre-B cells from rabbits heterozygous for *b* locus allotypes of kappa light chains express only one of the alternative alleles (14).

The *a*-locus determinants, markers for heavy-chain V-region immunoglobulin genes, are also expressed in rabbit pre-B cells (Gathings, W. E. et al., unpublished material). The present observations indicate that human pre-B cells also express products of immunoglobulin V genes. This evidence provides strong support for the concept that generation of antibody diversity begins at the pre-B-cell stage of development.

Finally, our evidence for the presence of Id<sup>+</sup> pre-B cells in these patients strongly suggests that the clonal oncogenic event that ultimately results in myeloma occurs within the bone marrow at the very earliest stage in B-cell development, and is not the consequence of antigen stimulation. This is in keeping with observations in mice indicating that T-cell malignancies invariably begin within the thymus (45) and in chickens that a B-cell malignancy originates within the bursal inductive microenvironment (46). Evidence from studies of malignancies of a variety of cell types suggests that the target cells for the oncogenic event are immature cells within the microenvironments in which they are normally induced to undergo specialized differentiation (47).

Compelling arguments have been made by Cairns (48) and Mintz (49) that the target cells for malignancies are invariably stem cells. Studies of mouse embryonic teratocarcinomas show that affected stem cells may continue to differentiate normally following the oncogenic event (50). The validity of these principles in humans is attested by observations of the myeloproliferative diseases, polycythemia vera, and chronic myelogenous leukemia. In the former, the hyperplastic line of erythroid cells derives from a stem cell that also gives rise to myelomonocytic and megakaryocytic cells but not the T and B cells (51). Transformation of an even more primitive stem cell apparently occurs in patients with chronic myelogenous leukemia, in which B cells, erythrocytes, myeloid cells, and megakaryocytic cells share the same clonal marker (52, 53). After treatment, chronic myelogenous leukemia may be replaced by a lymphoid leukemia involving the same clone (54); in two such individuals the malignant lymphoblasts were found to exhibit the pre-B-cell phenotype (55).<sup>2</sup>

In patients with acute lymphocytic leukemia of the pre-B-cell phenotype (21), the malignant pre-B cells apparently cannot differentiate to functional B lymphocytes or plasma cells. This study suggests a different scenario for myeloma. Pre-B cells or their stem-cell precursors are apparently the target for the oncogenic event, but they are not prevented from further differentiation and do not behave as malignant cells. In light of these observations, it seems likely that other B-cell malignancies, such as chronic lymphocytic leukemia, Burkitt's lymphoma, B-cell lymphomas, and Waldenström's macroglobulinemia, could reflect oncogenic events which occur at a stem cell level but differ in their effects on further differentiation of affected clones.

### Summary

IgA myeloma proteins of  $\kappa$ - and  $\lambda$ -types were isolated from two patients. These were used to produce and purify anti-idiotypic antibodies of both broad (myeloma-related) and narrow (individual myeloma) specificities. The anti-idiotypic antibodies were conjugated with fluorochromes and used as immunofluorescent probes to trace

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<sup>2</sup> Greaves, M. F., W. Verbi, L. B. Vogler, M. D. Cooper, R. Ellis, K. Ganeshaguru, V. Hoffbrand, G. Janossy and F. J. Bollum. Antigenic and enzymatic phenotypes of the pre-B subclass of acute lymphoblastic leukemia. Submitted for publication.

in the patients clonal expansion at different levels of B-cell differentiation. Our results (a) confirm that B lymphocyte precursors in IgA plasma-cell myelomas are involved in the malignant process, (b) show that B lymphocytes of the malignant clone include those expressing each of the major heavy-chain isotypes,  $\mu$ ,  $\delta$ ,  $\gamma$ , and  $\alpha$ , and (c) provide strong circumstantial evidence that pre-B-cell members of the malignant clone are also increased in frequency. T cells expressing idiotypic determinants were not detected. These findings argue that the initial oncogenic event may occur in a B-stem cell and is not influenced through stimulation by antigen. An interesting association was the increased frequency of related clones of B lymphocytes as detected by their reactivity with anti-idiotypic antibodies of broad specificity. Neither plasma cell nor pre-B-cell members of these related clones were increased in frequency. Anti-idiotypic antibodies or helper T cells reactive with myeloma-related idiotypes could be responsible for this phenomenon. We discuss other implications of these findings and speculate that all of the various phenotypes of B-lineage malignancies may result from oncogenic processes affecting stem cell targets.

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