Establishment of Idiotype Bearing B-Lymphocyte Clones From a Patient With Monoclonal Gammopathy

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B-lymphocyte clones bearing an idiotypic Ig structure, which could also be found on the serum M-component, have been isolated from peripheral blood of a patient with monoclonal gammopathy (MG) using an immune-rosetting technique and limiting dilution. The majority of the cells had the surface phenotype id+/CD19+/CD20−/CD21+ or id−/CD19+/CD20+/CD21+. Some of the clones originated from spontaneous outgrowth of lymphocytes and some were derived from in vitro Epstein-Barr virus-infected lymphocytes. Three of the B-lymphocyte clones and the patient's bone marrow plasma cells appeared to have clonal identity as shown by the same Ig heavy chain gene rearrangements in Southern blot analysis using a Jκ probe. The study further supports the notion that the peripheral blood of patients with MG contains B lymphocytes that belong to the tumor clone.

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MATERIALS AND METHODS

Patient

The patient was a 54-year-old woman (MO) with an M-component (IgGκ, λ) that had remained stable at the same serum concentration (about 15 g/L) for more than 14 years. At the time of the test, the BM contained 10% plasma cells. The hemoglobin level, s-calcium, s-albumin, and s-creatinine were normal. The patient was considered to have an MG of undetermined significance (MGUS).

Isolation of IgG and Preparation of F(ab′)2, Fragments

Isolation was performed in two steps, by ion exchange and by gel filtration using fast protein liquid chromatography (FPLC; Pharmacia, Uppsala, Sweden). The method has been described in detail elsewhere.2 The purity of the monoclonal IgG fraction was checked using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (PhastSystem; Pharmacia). F(ab′2), fragments of IgG were prepared by pepsin digestion (Sigma Chemical Co, St Louis, MO). To remove undigested IgG and Fc-fragments, the preparation was run through a Protein-A column (Pharmacia).

Preparation of Anti-id Monoclonal Antibodies (MoAbs)

Anti-id MoAbs were produced using a standard mouse hybridoma technique10 with the addition of a feeder cell layer, eliminating the need for subsequent cloning.10 A BALB/c mouse was immunized intraperitoneally with the IgG M-component in complete Freund's adjuvant. Spleen cells were fused with the mouse myeloma cell line SP2/0 and hybridoma supernatants were screened for the presence of antibodies against the patient's M-component in enzyme-linked immunosorbent assay (ELISA). One clone (no. 454) produced an IgG antibody that specifically bound to the F(ab′2), fragments of the M-component of patient MO. This antibody did not cross-react with pooled human normal IgG or with purified IgG myeloma proteins isolated from five other patients, including one who had an M-component of the IgGκ type, the same IgGκ subclass isotype as patient MO.

Coating of Erythrocytes with Anti-id Antibodies

Human erythrocytes were coated with the anti-id antibody (no. 454) using the CrCl3 method as previously described.11,12 One

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Establishment of Human B-Cell Lines

Immortalization by infection with EBV. Lymphocytes were isolated from heparinized venous blood from patient MO by Ficoll-Paque gradient centrifugation (Pharmacia). T cells were removed by rosetting with neuraminidase-treated sheep red blood cells (SRBCs) and separated on a Ficoll-Paque gradient. Non-T cells were then incubated for 60 minutes at 37°C with the transforming B95-8 strain of EBV. Cells were washed, incubated in RPMI 1640 (GIBCO Ltd, Paisley, Scotland) containing 10% fetal calf serum (FCS), in humidified air with 5% CO₂, at 37°C, and fed twice a week.

Spontaneous immortalization. PBL from patient MO were separated on a Ficoll-Paque gradient, depleted of T cells, and incubated in RPMI with 10% FCS as described above.

Selection and Enrichment of id⁺ cells

Two to 3 weeks after immortalization of the lymphocytes, a selection for id⁺ cells was made. Cells (4 x 10⁶) were washed and mixed in 10 mL RPMI/10% FCS with 400 μL of a 10% suspension of human erythrocytes coated with mouse anti-id antibodies. The mixture was spun at 500 rpm for 15 minutes at 4°C and further incubated at 4°C for 1 hour. Cells were then resuspended, layered on a Ficoll-Paque gradient (Pharmacia), and separated at 2,000 rpm for 20 minutes. The pelleted cells were collected and incubated in RPMI with 20% FCS in humidified air with 5% CO₂ at 37°C and fed twice a week.

After two weeks, the culture medium was changed to RPMI containing 10% FCS.

To achieve a further enrichment of id⁺ cells, a reselction of the cells was performed 2 weeks after the initial selection. The percentage of id⁺ cells in the enriched polyclonal cell cultures (as assessed by rosette assay [see below]) increased from initially 5% of the PB non-T lymphocytes to greater than 50% in the enriched population. Among PB B cells of five healthy controls, no such rosette-forming cells were detected.

Cloning of Cells

Cells that emerged after the enrichment procedures were cloned in 0.2 mL RPMI with 20% FCS in flat-bottom microplate wells (Nunclon, Roskilde, Denmark) (0.3 cells/well) together with 2 x 10⁶ heterologous irradiated (20 Gy) human lymphocytes/well. Microplates were kept in the incubator for 20 days with no feeding. Clones were then collected into tubes in 0.2 mL of medium and finally transferred into tissue culture flasks (Nunclon). During the first 10 days, the plates were kept horizontal but when the feeder layer had disappeared the plates were tilted so that growing cells were concentrated at one edge of the wells. Cells were seeded into 2,400 wells and 16 clones were obtained (2% cloning efficiency).

IFL

IFL assay was applied to determine heavy and light chain Ig isotypes of the cells. Before staining, the cells were incubated for 30 minutes at 37°C in serum-free medium and then washed three times at 37°C to remove adsorbed Ig. Cells were stained in direct IFL with fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragments of goat antihuman-IgG and -IgM antibodies (Tago, Burlingame, CA) and rabbit antihuman-κ and -λ light chain antibodies (DAKO A/S, Copenhagen, Denmark). Id-bearing (id⁺) cells were detected in indirect IFL using the anti-id antibody (no. 454) as the primary antibody. B-cell-related surface antigens were identified by indirect IFL using MoAbs against CD19, CD20, and CD21 (Coulter, Hialeah, FL). Staining for T, natural killer (NK), and monocyte surface structures was performed using indirect IFL with MoAbs against CD3, CD16, and CD14, respectively (Becton Dickinson, Mountain View, CA). In the second step, phycoerythrin-conjugated F(ab')₂ fragments of goat antiamoige IgG (Tago) were used. Cells were analyzed by flow cytometry (FACS; Becton Dickinson) at 480 nm with a flow rate of less than 300 cells/s.

Cell Morphology

The morphology of cloned cells was examined on cytocentrifuged May-Grünwald-Giemsa-stained slides using a Zeiss standard RA microscope in ordinary light, magnification x1,000.

Rosette Assay to Determine Surface id⁺ Cells

Surface-specific id⁺ cells were determined by a rosette assay with anti-id–coated human erythrocytes. Ten microliters of 0.5% suspension of anti-id–coated erythrocytes was mixed with 50 μL of lymphoid cells (4 x 10⁶/mL). The mixture was spun at 500 rpm for 15 minutes at 4°C and incubated at 4°C for 1 hour. The percentage of rosette-forming cells was determined by counting 400 cells in a Zeiss standard RA microscope.

Determination of the Idiotype of Igs

A competitive ELISA technique was used for detection of specific id⁺ Ig produced by the cell lines. Wells in micro-ELISA plates (Immulon 2; Dynatech Lab, Shantilly, VA) were coated at 4°C overnight with F(ab')₂ fragments of the M-component of the patient MO. Optimal concentration of anti-id antibodies was determined in an ELISA titration curve to give a value that was high but still within the logarithmic range, ie, without using an excess of antibody. Ten microliters of the titrated anti-id antibody (no. 454, 10 μg/mL) was incubated for 2 hours at 37°C with 90 μL of undiluted cell culture supernatant. The mixture was then added to the coated plates and incubated for 2 hours at 37°C. Wells were washed as above and 100 μL of alkaline phosphatase-conjugated goat antiamoige IgG (1:1,000) (Sigma) was added for 2 hours at 37°C. The plates were then reacted with alkaline phosphatase-conjugated goat antiamoige IgG (Sigma) followed by enzyme reaction using disodium p-nitrophenyl phosphate as previously described.² The absorbance was read at 405 nm after 30 to 60 minutes. The presence of Ig exhibiting the specific idiotype in the cell supernatant induced inhibition of binding of the murine antibody to the wells (ie, a decrease in the absorbance value) (see Results).

Epstein-Barr Nuclear Antigen (EBNA) Staining

Cell lines were stained for EBNA using the technique of Reedman and Klein.¹⁸

Isoelectric Focusing and Blotting

Isoelectric focusing was performed using a PhastSystem kit (Pharmacia) as described by Olsson et al.³ An amiphile with pH-gradient 3-9 (Phast Gelf IEF 3-9; Pharmacia) was used. Focused proteins were transferred to a polyviolide-difluoride membrane with 0.45-μm pore size (Immobilon Transfer Membrane; Millipore, Bedford, MA) by a method modified from that of Walker et al.¹⁴ After blocking with 3% bovine serum albumin for 45
minutes at RT, the membrane was incubated with the anti-id antibody (no. 454) (14 μg/mL) in buffered NaCl for 1 hour at RT, followed by washing three times for 15 minutes. A biotinylated horse antimouse antibody (30 μg/mL; Vector Lab, Burlingame, CA) was applied for 2 hours followed by color development with avidine-biotin peroxidase complex ( Vectastain ABC Kit; Vector Lab) according to the manufacturer’s description.

**DNA Preparation and Southern Blot Analysis**

BM mononuclear cells (BMMC) from patient MO were obtained by centrifugation of a heparinized BM sample on a Ficoll-Paque gradient (Pharmacia). T cells were removed from half of the BM sample by rosetting with neuraminidase-treated SRBC and were then separated on a Ficoll-Paque gradient.

DNA was extracted from the patient’s BM, the B-cell-enriched BM (B-BM), and the blood B-cell clones as previously described. Ten micrograms of high molecular weight DNA was digested with the restriction enzymes HindIII, EcoRI or BamHI (Amersham Int., Amersham, UK) for 8 hours at 37°C. The digested DNA was electrophoresed in 0.6% agarose gel at 40 V (2 V/cm) for 20 hours and transferred to nitrocellulose filter by the method of Southern. The human IgH-J, probe was kindly provided by Dr Philip Leder (Department of Genetics, Harvard Medical School, Boston, MA). It is a 5.6-kbp human genomic fragment. The probe was labeled with [32P] dCTP (deoxycytidine triphosphate) by nick translation. Hybridization and washing were as described by Wahl et al. The hybridized blots were washed and exposed to film ( Fuji Photo Film Lab) according to the manufacturer’s description.

**RESULTS**

**Phenotypic Characteristics of the Clones**

Most cloned cells had a lymphoblastoid/lymphoplasmocytoid appearance, but small lymphocytes were also seen. The proportion of cells with mature plasma cell morphology was less than 5%. The cells were growing rapidly with a doubling time of about 2 days. A slight expression of NK cell morphology (large granular lymphocytes) was found. Positivity for CD16 was found on some clones, but no cells exhibited a low frequency (<20%) of CD21. A slight expression of CD21 was found. Five clones (nos. 1, 3, 5, 10, and 12) had a CD21 expression of <20% (no. 10), indicating a B-cell origin.

A varying degree of CD21 expression was found. Most cloned cells had a CD21 expression of 25-80%. Eight clones had greater than 50% cells stained in IFL with the anti-id antibody (no. 454) on the cell surface of lymphoid cell lines.

As expected, all in vitro EBV-transformed clones were EBNA-positive (Table 2). Spontaneously growing cells were also found to be EBNA-positive, indicating that they emerged either from an in vivo EBV-infected cell or were indirectly infected by a virus released from another cell. Due to loss of some clones, neither EBNA staining nor surface membrane receptor staining could be performed on all clones.

### Table 2. Characteristics of the Cell Clones

| Clone No. | s-id<sup>*</sup> Cells<sup>+</sup> s-id<sup>*</sup> Cells<sup>+</sup> % Inhibition in ELISA of Supernatant Staining |
|-----------|-------------------------------------------------|-------------------------------------------------|
| 1         | IgM<sub>K</sub> 79 — 93 Pos                    |
| 2         | IgM<sub>K</sub> 6 — 34 Pos                     |
| 3         | IgM<sub>K</sub> 85 — 94 Pos                    |
| 4         | IgM<sub>K</sub> 75 — 95 Pos                    |
| 5         | IgM<sub>K</sub> 80 — 90 Pos                    |
| 6         | IgG<sub>λ</sub> 72 — 80 Pos                    |
| 7         | IgG<sub>λ</sub> 83 — 71 Pos                    |
| 8         | IgG<sub>κ</sub> 48 72 20 Pos                   |
| 9         | IgM<sub>K</sub> 5 — 0 Pos                     |
| 10        | IgM<sub>K</sub> 4 — 0 Pos                     |
| Control   | EBV-transformed nonenriched polyclonal cells    |
|           | from a nonrelevant myeloma patient             |
|           | IgM 0 — 1 Pos                                 |

Abbreviations: s-surface; —-— not done; Pos, positive.

<sup>*</sup>Percent cells forming rosettes with erythrocytes coated with the anti-id MoAb (no. 454). Rosette formation was shown to be specifically inhibitable by the patient’s M-component.

The presence of the specific idiotype (identified by the murine MoAb, no. 454) on the cell surface of lymphoid cell lines and on secreted Ig from isolated clones was tested in IFL, in a rosette-forming assay and ELISA (Table 2). Normally, a monoclonal B-cell line shows a dynamic variation in the expression of surface structures. A cell line showing greater than 70% cells forming specific rosettes or greater than 50% cells stained in IFL with the anti-id antibody (no. 454) were considered to be monoclonal id<sup>s-id+</sup> cell cultures (Table 2). Eight clones had greater than 50% id<sup>s-id+</sup> cells in IFL (nos. 1, 3, 4, 5, 6, 7, 11, and 12). The binding specificity of the Ig produced by the clones was confirmed by inhibition experiment in ELISA. Two of the clones (nos. 11 and 12) also formed rosettes. Two clones (nos. 14 and 16) were not studied in IFL but formed a high percentage of rosettes and their supernatants showed a high degree of specific inhibition in ELISA (> 80%). Thus,
10 clones (nos. 1, 3, 4, 5, 6, 7, 11, 12, 14, and 16) fulfilled the criteria of id+ clones. Clone no. 15 should probably also be considered as id+ clones. Four of the clones (nos. 2, 9, 10, and 13) were not id+ on neither the cell surface nor the IgM produced into the supernatant. The remaining clones (nos. 8 and 15) had some id+ characteristics. The isotype could be determined in 12 clones. Three clones (nos. 6, 7, and 8) were of the IgG type and the remaining were of the IgM type. Within each clone, the percentage of cells positively stained by the anti-heavy chain antibody and the anti-id antibody (no. 454), respectively, were always similar. The dual expression of the isotype was confirmed by two-color IFL analysis (not shown). The majority of the clones expressed \( \kappa \)-chains, similar to the serum M-component. Three id+ clones (nos. 6, 7, and 11) expressed \( \lambda \)-chains.

EBV-transformed nonenriched control cells from another patient with an IgG myeloma did not express the specific idiotype measured in IFL and in ELISA.

Inhibition curves of representative supernatants from clones no. 6, 7, and 8 are shown in Fig 1. Inhibition of the binding of the anti-id antibody (no. 454) to F(ab')\_2 of the autologous M-component was dose-dependent. The supernatants of clones no. 6 and 7 had a similar inhibitory capacity as the autologous M-component, whereas the inhibitory capacity of clone no. 8 was low. A nonrelevant isotype-matched IgG M-component showed no inhibitory capability, as was the case for polyclonal IgG.

**Isoelectric Focusing**

Isoelectric focusing of supernatants from the IgG-positive clones no. 6, 7, and 8 followed by blotting and staining with anti-id antibodies (no. 454) is shown in Fig 2. The clones showed two to four close bands, indicating monoclonality. Clones no. 6 and 7 had a high percentage of specific id+ cells and the supernatants induced a high degree of inhibition (Table 2). Ig of these clones had an isoelectric point (pl) identical or very close to that of the M-component of the patient. In contrast, the Ig of clone no. 8 had a different pl, in accordance with a low percentage of specific id+ cells and a low degree of inhibition of binding of the no. 454 antibody to the MO M-component induced by the cell supernatant (Table 2).

**Southern Blot Analysis of the Cell Clones**

The results with the \( J_\kappa \) probe and HindIII enzyme are shown in Fig 3. In addition to germline DNA, weak \( J_\kappa \) bands were detected in the patient's B-BM, but not in the nonenriched fraction (BM), indicating the presence of a small monoclonal B-cell fraction (= 10%). The DNA of clones no. 5, 6, and 7 appeared to have \( J_\kappa \) rearrangement patterns identical to the specific one detected in B-BM. These clones had a high percentage of specific id+ cells and their supernatants induced a high degree of inhibition in ELISA (Table 2). The IgG of clones no. 6 and 7 had also the same isoelectric point as the serum M-component (Fig 2). The other clones had individual \( J_\kappa \) rearrangement patterns. They were all clearly different from the unique pattern of the B-BM sample and of clones no. 5, 6, and 7. Cell culture no. 3 had more than two \( J_\kappa \) bands, indicating that it was not monoclonal. Identical results were obtained for the three clones no. 5, 6, and 7 when DNA was digested with EcoRI (Fig 4) or BamHI (not shown) and hybridized with the same \( J_\kappa \) probe. Due to lack of material, the DNA from the BM or B-BM could only be digested with HindIII enzyme.

**DISCUSSION**

It is of theoretical as well as clinical interest to characterize the clonal B-cell population in MG. Evidence has accumulated indicating that MG may be differentiating B-cell tumors originating from an early B cell.\(^7\) More information about the disease would be obtained if such cells could be isolated. By using molecular methods it might be possible to trace the progenitor cells.

The blood of MG patients might contain id-bearing lymphoid cells. Thus, PB should be a convenient source for obtaining cells belonging to the tumor clone. For this purpose, it might be preferable to study patients with MGUS because these patients have a stable condition that does not require therapy. However, id+ cells in the blood of MGUS patients do not constitute more than 1% to 4% of the blood B-cell compartment.\(^22\) Thus, isolation of a specific id+ cell fraction from blood might be difficult.

In the present study, B-lymphocyte clones bearing an idiotype similar to that of the serum M-component have been isolated from the blood of a patient with MGUS. After enrichment by rosette formation, an efficient and reproducible cloning was applied that enabled the establishment of clones with specific id on the cell membrane. The rosette technique can be used for enrichment of cells even if present at frequency of \( 10^{-6} \).\(^{26} \) Cloning of 0.3 cells/well eliminated a potential overgrowth of more rapidly growing id+ cells. In our study, clones were obtained in 10 wells from
Fig 2. Isoelectric focusing and blotting of supernatants from three IgG clones (nos. 6, 7, and 8) and the serum M-component of patient MO. Focused proteins were transferred to a polyvinylidene difluoride membrane, incubated with the anti-id antibody (no. 454), followed by color development with biotin-avidin peroxidase complex. The isoelectric point (pl) of the monoclonal protein (indicated by two to four close bands) can be determined from the pH scale.

Fig 3. Ig heavy-chain gene rearrangement analysis of the patient's BM, T-cell depleted BM (B-BM), and B-cell clones (nos. 1 through 12) with a cloned human Jh gene fragment as a probe. DNA was digested with HindIII enzyme. Dashes correspond to the positions of DNA size standards with sizes shown in kilobases (kb). The germline band is indicated by a solid arrowhead. The original DNA application site is indicated by an open arrowhead.
in vitro EBV-infected cells and in six wells from spontaneously growing cells out of a total of 2,400 wells. The majority of the clones were of IgM type. This might be due to a lack of Ig class switch by most of id+ PBL. However, the method used for selection of id+ cells might have preference for IgM+ cells. It is still an open question whether EBV preferentially infects cells that express certain Ig isotypes.2,28

A previous attempt to establish id+ B-cell lines from patients with MG was not successful. In the study by Clofent et al,29 cells were cloned by limiting dilution without prior enrichment for id+ cells and no id+ cell lines were obtained. We assume that the enrichment for id+ cells may be an important step. Another approach was used by Grogan et al,28 who established long-term pre-B cell cultures from myeloma BM samples. Although these pre-B cells had some characteristics of precursor cells to the malignant plasma cells, a definite proof (ie, identical Ig gene rearrangements and specific id expression of both pre-B and plasma cells) was not given.

Southern blot with JH probe of DNA from B-BM gave a specific pattern of Ig gene rearrangements. It might be assumed that the rearranged bands in the BM sample emerged from the plasma cell clone constituting 10% of the total cell count. The detection limit with our technique is about 10%. One IgM-κ clone (no. 5) had Ig heavy chain gene rearrangement that appeared to be identical to that of the BM cells. A high percentage of cells in this clone had surface id and secreted Ig with the specific idiotype. Thus, clone no. 5 is probably derived from a pre-switch B lymphocyte that might be part of the same B-cell clone as the id+ BM plasma cells. Previous studies using IFL have indicated that the tumor cell clone in MG patients might comprise IgM+ pre-switch cells.31,32

Two IgG+ clones (nos. 6 and 7) that showed a high number of id+ cells as well as a high inhibition in ELISA have rearranged JH bands that appeared to be identical to that of the BM plasma cells and to clone no. 5. The JH bands of clones no. 5, 6, and 7 may not necessarily be identical to that of the BM plasma cells. However, it is assumed that these cell clones originate from a cell that belongs to the same clone as the id+ BM plasma cells. The finding that clones no. 6 and 7 expressed λ-chains in contrast to the M-component, which had κ-specificity, and to clone no. 5, which expressed IgM-λ, might indicate that the tumor cell compartment may comprise not only B cells and plasma cells with rearranged heavy and light chain genes but also B cells that have not yet rearranged their light chain genes but have rearranged their heavy chain genes. Thus, cells with identical heavy chain rearrangement but different light chain isotypes may emerge.33 This assumption is supported by results from Hata et al,31 who described myeloma cells with expression of germline Ig light chain genes. Saltman et al34 reported one case of concomitant chronic lymphocytic leukemia (CLL) of IgM-κ type and multiple myeloma of IgA-λ type where identical Ig heavy chain rearrangements in the blood and BM were found, indicating a single B-cell progenitor. In addition, Miyamura et al35 described a patient with a large-cell lymphoma and CLL expressing different light chain phenotypes but identical Ig heavy chain rearrangements. Grogan et al28 and Epstein et al36 showed the presence of myelomonocytic, megakaryocytic, and erythroid surface markers on myeloma plasma cells, further supporting the notion that the tumor might originate at the pluripotent stem cell level.

It is conceivable that each of the isolated id-expressing clones has one of the following origins: (1) A B cell that may belong to the clone that produces the serum M-component. (2) A B cell with an identical idiotope (detected by the binding to the mouse anti-id no. 454) but with a Ig gene rearrangement different from the tumor clone. Miller et al37 produced 199 anti-idiotypic Ig antibodies against 199 B-cell tumors. Twenty of them reacted with 33% of B-cell tumors, showing the existence of shared idiotopes in B-cell tumors. (3) A B cell that belongs to an anti-anti-idiotype (Ab2) producing clone that has emerged as a response to anti-idiotypic antibodies (Ab1) according to the network hypothesis.38 We have recently shown that this particular patient had a high production of anti-idiotypic antibodies (Ab1). A specific binding of id+ cells to purified Ab1 would strengthen this assumption. Such experiments are in progress.

In general, malignancy is a result of a sequence of genetic events that takes place during differentiation.45,46 None of these events is sufficient by itself to cause the malignant phenotype. The facts that our cell lines shared idiotype with the tumor plasma cell clone and that they had the same JH rearrangement supports the assumption that they originated from the same progenitor cell. However, additional
events may be required to develop a fully “malignant” phenotype of the cells.

In conclusion, this study further strengthens the assumption that there are idiotype-bearing early B lymphocytes in the blood of MG patients and that these cells may belong to the Ig-producing tumor clone. These isolated id+ B-lymphocyte clones might be useful tools in studies of the specific and nonspecific regulation of the B-cell clone.

REFERENCES


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