DOUBLE ISOTYPE PRODUCTION BY A NEOPLASTIC B CELL LINE

I. Cellular and Biochemical Characterization of a Variant of BCL_1 that Expresses and Secretes Both IgM and IgG1

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The humoral immune response is characterized by the synthesis and secretion of IgM and subsequently IgG antibody (1, 2). The precursors for IgG-secreting cells are the descendants of cells that initially express or secrete IgM (2, 3). The switch from IgM to IgG synthesis can occur in both small lymphocytes (4–9) and plasma cells (3). The small lymphocytes that have undergone this switch are thought to be memory cells that give rise to the vast majority of IgG-secreting cells in the secondary antibody response (10). At the molecular level, two basic mechanisms have been suggested for switching: (*a*) deletion of DNA 5′ to the expressed C_H either by intrachromatid (11) or sister chromatid (12) recombination or (*b*) RNA splicing of a single nuclear transcript containing more than one C_H region (13, 14).

During the process of antigen- or mitogen-induced switching, B cells expressing two isotypes (e.g., IgM and IgG) have been described. The basis of simultaneous production of two isotypes by a single cell is not well understood. It is possible that DNA rearrangements occur in terminally differentiating cells, whereas RNA splicing occurs in memory cells or in cells producing two isotypes.

The vast majority of studies involving switching have used switch variants of myeloma, hybridoma, and virally transformed cells that have lost their capacity to synthesize and/or secrete one isotype and then express or secrete another. In many cases, it has been found that switching in these neoplastic cells occurs by the activation of two chromosomes (15) or by the use of switch regions outside of those normally used by nonneoplastic cells (16).

In this study we describe the isolation of a stable switch variant of the in vitro–adapted BCL_1 cell line ($BCL_1.B_1$). BCL_1 was the first B cell leukemia described in mice, and was shown to express large amounts of surface IgM and a trace

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amount of surface IgD (17); both isotypes bear the same idiotype (18). A cell line was established ($BCL_1.B_1$) that expresses surface IgM (sIgM)[1] and very small amounts of sIgD, and these cells can be induced to secrete IgM after the addition of LPS (19). All the cells of the switch variant synthesize, express on their surface, and secrete both IgM and IgG1.

Materials and Methods

Cell Lines. The in vitro–adapted $BCL_1.B_1$ cell line was kindly provided by Dr. Samuel Strober, Department of Medicine, Stanford University, Stanford, CA; it was derived by Gronowicz et al. (19). The cell line was maintained in RPMI 1640 medium supplemented with 10% FCS, 5×10^{-5} M 2-ME, 2 mM glutamine, 10 mM nonessential amino acids, 1 mM sodium pyruvate, 100 μg/ml penicillin, 100 U/ml streptomycin, and 5 μg/ml gentamycin. Hybridoma cell lines 5A5, 9B12, 13C8, 6A2 which secrete IgM, IgG1, IgG2b, and IgG3, respectively, have been described previously (20, 21), and were gifts from Drs. E. Hansen and M. Norgard, Department of Microbiology, UTHSCD.

Cell Cloning. The $BCL_1.B_1$ line was cloned by limiting dilution in a 96-well culture plate (Costar, Cambridge, MA), as described by Brooks et al. (22). The cloning medium is the same as the growth medium, except that 25% WEHI-274 supernatant (SN) (from day 4 cultures) was added, and the concentration of FCS was adjusted to 15%. The culture SN was collected from the wells 3–4 wk after cloning and were assayed for the presence of IgG and IgM by a solid-phase RIA.

Subsequent subcloning of $BCL_1.2.58$ (see Results section for this subclone) was performed in the same way as the initial cloning procedure, except that single cells were deposited into single wells of a microtiter plate by the FACS III.

Preparations of Class and Subclass-Specific Antibodies and Their Conjugation to Sepharose. Monospecific affinity-purified rabbit antibodies against mouse Ig (RAMIg), μ (RAMμ), γ (RAMγ), γ1 (RAMγ1), γ2a (RAMγ2a), γ2b (RAMγ2b), γ3 (RAMγ3), OVA (RAOVA), goat Ig (RAGIg), L chain (RAML), δ (RAMδ), and goat antibodies against mouse μ (GAMμ), and rabbit Ig (GARIg) were prepared as described previously (23). Rabbit anti-BCL_1 idiotype (RABCL_1 Id) serum was prepared and characterized according to Vitetta et al. (18). $F(ab')_2$ fragments of the affinity-purified antibodies were prepared by pepsin digestion (24). Antibodies were conjugated to Sepharose 4B as described previously (23). The Sepharose contained 1 mg of antibody per milliliter of packed Sepharose.

RIA for the Quantification of IgM and IgG. A solid-phase RIA was used to determine the concentrations of IgM and IgG in the culture SN (23, 25). Standard curves using purified myeloma or hybridoma proteins were included in each assay. The sensitivity of these RIAs is 1 ng Ig per ml of SN.

Biosynthetic Labeling. 10^7 cells in log phase were harvested, washed, and resuspended in 1 ml of labeling medium containing spinner salt solution supplemented with 5% dialyzed FCS, 1.8×10^{-4} M $CaCl_2$, MEM vitamin solution, and all essential and nonessential amino acids, except methionine. 1 mCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) was added and the culture SNs were collected 6 h later. The SNs were dialyzed and subjected to immunoprecipitation using the appropriate antibodies.

Surface Radioiodination. 10^7 cells were harvested and washed in PBS and were iodinated as described previously (26). Iodinated cells were washed three times with PBS, lysed in 0.5% NP-40 in PBS at 4°C for 10 min. The cell lysates were collected after removal of the nuclei by centrifugation.

Immunoprecipitation. Aliquots of cell lysates from radioiodinated cells (2.5×10^6 cell equivalents), or culture SN from biosynthetically-labeled cells (10^6 cell equivalents) were incubated for 15 min at 37°C with 10 μg of RAOVA that had been passed twice over a

[1] *Abbreviations used in this paper:* BSS, balanced salt solution; MFI, mean fluorescence intensity; MOPS, 3-(*N*-morpholino) propanesulfonic acid; RAOVA, rabbit antibodies against OVA; RAMIg, rabbit antibodies against mouse Ig; s, surface; SN, supernatant.

Sepharose-mouse IgG column. 200 μl of 20% vol/vol Sepharose-GARIg was added for 15 min at 37° or 60 min at 4°C. Sepharose was recovered by centrifugation. The precleared lysate was then treated with 10 μg of the appropriate rabbit antibody for 15 min at 37°C, followed by Sepharose-GARIg for 15 min at 25°C. Sepharose was recovered by centrifugation and washed three times with PBS. The radioactive Ig bound to the washed Sepharose was extracted with SDS and subjected to SDS-PAGE. A portion of the lysate from radioiodinated cells was treated with 200 μl of 10% *Staphylococcus aureus*. The samples were centrifuged and the SNs were subjected to immunoprecipitation as described above.

SDS-PAGE Analysis. For the immunoprecipitation of samples prepared from SN of biosynthetically-labeled cells, 7.5% acrylamide tube gels were used to detect the secreted IgM and IgG. The samples were electrophoresed under reducing conditions for 16 h at 5 mA per tube. The gels were flushed from the tube and crushed into 70 fractions with a Savant gel crusher. The radioactivity in each fraction was determined by liquid scintillation counting. For the samples extracted from radioiodinated cells, we used a 10% slab gel in a Tris buffer system. The gels were electrophoresed at 150 V for 4 h. The gels were then fixed, dried, and exposed to x-ray film (XAR-5, Eastman Kodak Co., Rochester, NY) with intensifying screen.

Immunofluorescence. 10^6 cells were harvested and washed in balanced salt solution (BSS) containing 5% FCS and 10 mM NaN_3. 10 μg of primary antibodies were added, and cells were incubated at 4°C for 15 min. followed by three washes in medium. 10 μl of FITC-conjugated secondary antibodies were then added and cells were incubated at 4°C for 15 min. Cells were washed three times and suspended in 1 ml of medium. Cells were analyzed for the percent positive cells and mean fluorescence intensity (MFI) using the FACS III (Becton Dickinson, Mountain View, CA).

DNA Isolation and Southern Hybridization. High molecular weight DNA was isolated as described (27). 10 μg of DNA from each sample was digested with several restriction enzymes (Boehringer Mannheim Biochemicals, Indianapolis, Indiana), and was electrophoresed in a 1% agarose gel at 40 V for 18 h. The gel was denatured, transferred to a nitrocellulose filter (Schleicher & Schuell, Inc., Keene, NH) in 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na_3 citrate) (28), and the filter was hybridized for 18 h at 42°C in Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA) containing 5× SSC, 50% deionized formamide, 100 μg/ml herring sperm DNA, 30 μg/ml polycytidylic acid with 2×10^6 cpm/ml of ^{32}P-labeled probe (described below). Filters were washed in 3× SSC plus 0.1% SDS at room temperature, followed by 0.1× SSC and 0.1% SDS at 42°C for 1 h. They were then dried and exposed to x-ray film (XAR-5, Eastman Kodak Co.) at −70°C for 3 days with intensifying screens.

RNA Isolation and Northern Analysis. Total cellular RNA was isolated from the hybridoma cell lines and from the $BCL_1.B_1$ parent line and the $BCL_1.2.58$ variant according to Chirgwin et al. (29). 2.5 μg of the appropriate control RNA (isolated from hybridoma cell lines secreting either IgM, IgG1, IgG2b, or IgG3) or 15 μg of RNA isolated from $BCL_1.B_1$ and $BCL_1.2.58$, were dissolved in denaturing buffer containing 6% formaldehyde, 50% deionized formamide, and 40 mM 3-(*N*-Morpholino) propanesulfonic acid (MOPS) buffer. Samples were heated to 90°C for 2 min, then chilled and electrophoresed in 2% agarose gels in MOPS buffer and 6% formaldehyde. After 6 h of electrophoresis at 175 V or 20 h at 50 V, the gels were washed three times with H_2O at 65°C, and were stained with ethidium bromide to identify the 28S and 18S (eukaryotic), 23S and 16S (bacterial) ribosomal RNAs, which were used as molecular weight markers. The gel was transferred to nitrocellulose filters in 10X SSC, and filters were hybridized for 16 h with $1–10 \times 10^6$ cpm/ml of ^{32}P-labeled DNA probe in a manner analogous to Southern hybridization, except 30 μg/ml of polyadenylic acid was added. Filters were washed in 3× SSC in 0.1% SDS at room temperature followed by 0.1× SSC in 0.1% SDS at 42°C for 1 h before drying and exposure to x-ray film (XAR-5, Eastman Kodak Co.) at −70°C for 1–3 d with intensifying screen.

Preparation of DNA Probes. Genomic (J_H3-J_H4 and γ3) and cDNA constant region probes (γ1, γ2b, μ) were prepared by isolation of fragments prepared by treatment of the

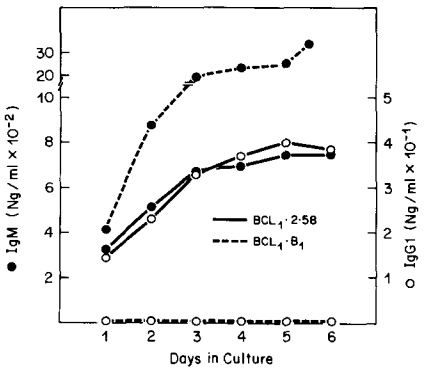

FIGURE 1. Secretion of IgG and/or IgM by $BCL_1.2.58$ cells and $BCL_1.B_1$. 5×10^4 cells were cultured in 200 μl of medium. Culture SN were collected for 6 consecutive days and were assayed for the presence of IgM and IgG subclasses. Each point is the average of triplicate wells. (●) IgM; (○) IgG1; (—) $BCL_1.2.58$; (---) $BCL_1.B_1$.

DNA with endonucleases from previously characterized recombinant clones (see below). Fragments encoding CH_3 domains of the IgG subclasses show the least homology and were chosen to avoid crosshybridization. The restriction digests were electrophoresed in 7.5% acrylamide gels containing 25% glycerol, and the appropriate fragments were isolated by elution (30). The γ1 probe is a 481-bp Hinc II/Hinf I fragment from plasmid pH21 (31) that includes 51 residues of the CH_2 domain and the entire CH_3 domain. The γ2b probe is a 320-bp Sac I/Sac I fragment from plasmid pγ2b(11)[7] (32) that includes almost the entire CH_3 domain. The γ3 probe is a 473-bp Bste II/Sph I fragment from plasmid pDR1 (33) that includes 235 bp of the CH_3 domain and the entire 3′ untranslated region. The Cμ probe is a mixture of 401-bp and 567-bp Pst I/Pst I fragment from p104Eμ12.39 (34) that includes 60 residues of the CH_2 domain and the entire CH_3, CH_4, and 3′ untranslated region. The J_H-specific probe is a 2-kb BamHI/EcoRI fragment (P. Tucker, personal communication), which includes J_{H3}, J_{H4}, and ~1.5 kb downstream. DNA probes were labeled with α-[^{32}P]dCTP by nick translation (35) to a specific activity of 5–10 × 10^7 cpm/μg.

Results

Cells from $BCL_1.2.58$ Clone Secrete both IgM and IgG1. In the initial cloning, the culture SN from the growth-positive wells were assayed for IgM and IgG by a solid-phase RIA. 840 clones were screened and 1 secreted both IgM and IgG. This clone was selected, expanded, and recloned at 1 cell/well by limiting dilution. A subclone ($BCL_1.2.58$) was selected and subjected to further characterization. Another subclone, $BCL_1.2.62$, which secreted only IgM, was also selected. 5×10^4 $BCL_1.2.58$ cells in 200 μl of medium were cultured in 96-well microtiter plates. SN from $BCL_1.B_1$ (the parent clone) and $BCL_1.2.58$ cells cultured in microtiter plates were collected for 6 consecutive days and assayed for the presence of IgM and IgG1 by RIA. Fig. 1 shows the kinetics of accumulation of IgM and IgG1 in the medium. Levels of IgM and IgG1 increased in the culture SN of $BCL_1.2.58$ for ~3 d. The ratio of secreted IgM to secreted IgG1 was constant (19:1) throughout the 6 d of the culture period. No IgG2a, IgG2b, or IgG3 was detected in the culture SN (<1 ng/ml). IgM, but not IgG1, was detected in the culture SN of the parent $BCL_1.B_1$ line (Fig. 1).

To confirm that the $BCL_1.2.58$ cells secrete IgG1, the cells were pulsed with [^{35}S]methionine and the SN was precipitated by RAMIg and Sepharose-GARIg.

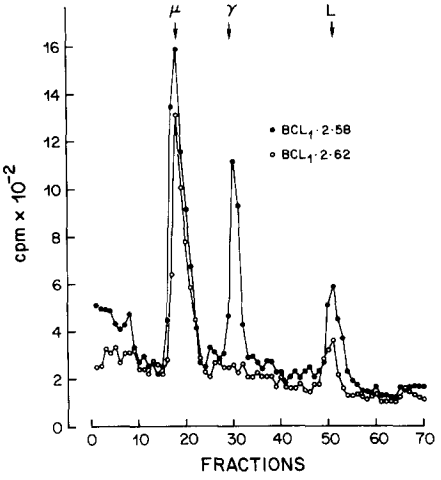

FIGURE 2. SDS-PAGE [^{35}S]methionine-labeled Igs secreted by BCL_1.2.58 and BCL_1.2.62 cells. Cells were incubated with the isotope and SNs were treated with RAMIg followed by GARIg-Sepharose. The Sepharose was pelleted, and the absorbed radioactivity was extracted and analyzed by SDS-PAGE under reducing conditions in a tube gel system. The gels were crushed and each fraction was counted in liquid scintillation counter. Iodinated MOPC-104E (IgM) and MOPC-21 (IgG_1) myeloma proteins were included as molecular weight markers. Arrows indicate the positions for μ, γ, and light chains.

The absorbed radioactivity was eluted and analyzed by SDS-PAGE. As shown in Fig. 2, under reducing conditions, biosynthetically labeled Ig secreted by BCL_1.2.58 cells showed peaks characteristic of normal secreted forms of μ, γ, and L chains. In contrast, analysis of Ig secreted by the BCL_1.2.62 cells (which secrete only IgM) revealed μ and L chain peaks only. These results support the RIA data and indicate that BCL_1.2.58 cells secrete IgM and IgG.

We noticed that there is a discrepancy in the IgM to IgG1 ratio as assayed by RIA vs. immunoprecipitation. This discrepancy is most likely due to the fact that the RIA is independent of antibody avidity and generally approaches completion, whereas immunoprecipitation does not.

To determine whether every cell from the BCL_1.2.58 secreted both IgM and IgG1, a single-cell cloning assay was performed. Cells were sorted by the FACS and single cells were deposited in 960 separate wells. Cells grew in 307 wells and all growth-positive wells contained secreted IgM and IgG1, as determined by RIA. These data indicate that every cell from the BCL_1.2.58 clone secretes both IgM and IgG1.

Surface Phenotyping of the BCL_1.2.58 Cells. To determine which sIgs were expressed on the BCL_1.2.58 clone, cells were harvested, washed, and reacted with GAμ, GAδ, F(ab')$_2$ RAMγ, or RABCL_1Id, respectively, followed by incubation with F(ab')$_2$ fragments of FITC-RAGIg or FITC-GARIg as required. The stained cells were analyzed on the FACS. F(ab')$_2$ fragments of normal rabbit or intact RAOVA antibodies or normal goat IgG were included as controls for the primary antibodies. Fig. 3 shows the fluorescence profiles of BCL_1.2.58 cells stained with GAMμ and F(ab')$_2$ fragment of RAMγ, respectively. These and other immunofluorescence data are summarized in Table I. ~95% of the BCL_1.2.58 cells express sIgM, sIgG, and the BCL_1 idiotype. In contrast,

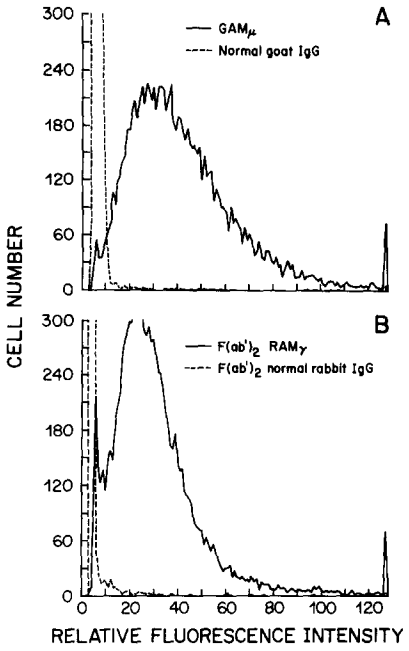

FIGURE 3. $BCL_1.2.58$ cells express both sIgM and sIgG as determined by analysis on the FACS. 10^6 $BCL_1.2.58$ were harvested, washed, and reacted with: (*A*) GAMμ (—) or normal goat IgG (---); (*B*) $F(ab')_2$ fragments of RAMγ (—) or normal rabbit IgG (---), respectively, followed by incubation with $F(ab')_2$ fragment of FITC-RAGIg (*A*) or FITC-GARIg (*B*); they were then analyzed on the FACS III. 10^5 cells were analyzed for each sample and the data were expressed as histograms.

TABLE I

Cell Surface Phenotype of $BCL_1.B_1$ and Its Derivatives

Antibody	Cells			
	$BCL_1.B_1$	$BCL_1.2.58$	$BCL_1.2.62$	Spleen
GAMμ	98.7	94.1	90.6	40.0
(MFI‡)	(75.3)	(59.3)	(55.4)	(29.0)
GAMδ	5.6	5.0	6.0	41.6
Normal goat IgG (control)	2.8	3.8	3.5	6.2
$F(ab')_2$ fragment of RAMγ	8.9	93.0	5.4	6.1
(MFI)	—	(39.0)	—	—
$F(ab')_2$ fragment of normal rabbit IgG (control)	4.4	4.3	4.0	3.3
$RABCL_1Id$	94.3	95.6	93.1	7.9
(MFI)	(72.1)	(75.4)	(76.9)	—
RAOVA (control)	5.3	4.6	4.5	5.4

Data are expressed as % positive cells reacting with the indicated antibodies. 10^6 cells were harvested, washed, and reacted with 10 μg of GAMμ, GAMδ, $F(ab')_2$ fragment of RAMγ, $RABCL_1$ Id, normal goat IgG, $F(ab')_2$ fragment of normal rabbit IgG or RAOVA, respectively. The cells were then washed and were incubated with 10 μl of FITC-GARIg or $F(ab')_2$ fragment of FITC-RAGIg, and they were then analyzed by a FACS III. All the data are an average of three determinations.

* MFI, mean fluorescence intensity.

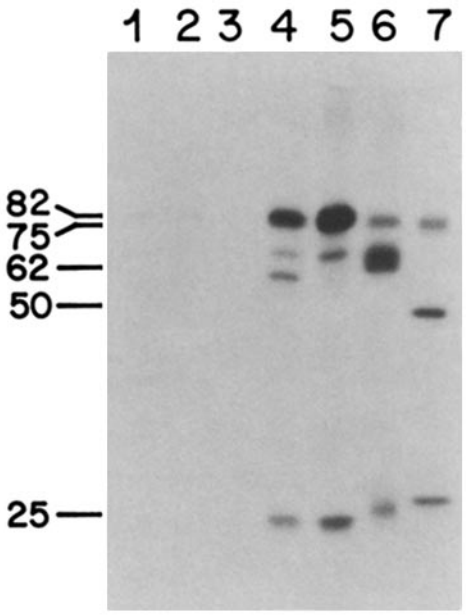

FIGURE 4. SDS-PAGE of Ig immunoprecipitated from cell lysates of radioiodinated BCL_1.2.58 cells, BCL_1.2.62 cells, and spleen cells. 10^7 cells were radiolabeled with ^{125}I. The cell lysates were treated with RAOVA (Lane *1*, *2*, and *3*) or RAML (lane *4*, *5*, and *6*) and GARIg-Sepharose. Eluates of the Sepharose were analyzed by SDS-PAGE. Lane *1* and *4* were cell lysates from BCL_1.2.58 cells, lane *2* and *5* were cell lysates from BCL_1.2.62 cells, and lane *3* and *6* were cell lysates of spleen cells. Lane *7* contained iodinated MOPC-104E (IgM) and MOPC-21 (IgG1), as molecular weight markers for μ, γ, and L chains. kD markers are shown in left margin.

BCL_1.2.62, (which secretes only IgM), lacks sIgG. Spleen cells were included as an additional control and the values were within the ranges reported previously (36, 37). Like the parent line, BCL_1.2.58 and BCL_1.2.62 display borderline staining for sIgD.

Analysis of sIg by Radioiodination. To confirm the presence of both sIgG and sIgM on the BCL_1.2.58 cells, and to exclude the possibility of cytophilic attachment of IgG, cells were radioiodinated, the lysates were precipitated with the appropriate antibodies, and were analyzed by SDS-PAGE. As shown in Fig. 4, RAOVA did not precipitate radioactivity from cell lysates of BCL_1.2.58 cells (lane *1*), BCL_1.2.62 cells (lane *2*), or spleen cells (lane *3*). However, RAML precipitates from BCL_1.2.58 (lane *4*) electrophoresed under reducing conditions displayed μ, γ, and L chain bands. The μ and γ chains have the characteristic high molecular weights of cell surface μ and γ chains, i.e., 82 and 62 kD, respectively. In contrast, the BCL_1.2.62 (lane *5*) cell line lacks sIgG. Normal spleen cells (lane *6*) express sIgM and sIgD. The 70-kD band observed in lanes *4* and *5* was not seen in subsequent experiments when 0.5% NP-40 was incorporated into the washing buffer.

To prove that the 62-kD molecular weight species was a γ chain, the lysates were first treated with GAMμ-Sepharose and the SNs were precipitated with RAMγ or RABCL_1 Id followed by absorption of the immune complexes to GARIg-Sepharose. The immune complexes were eluted and electrophoresed

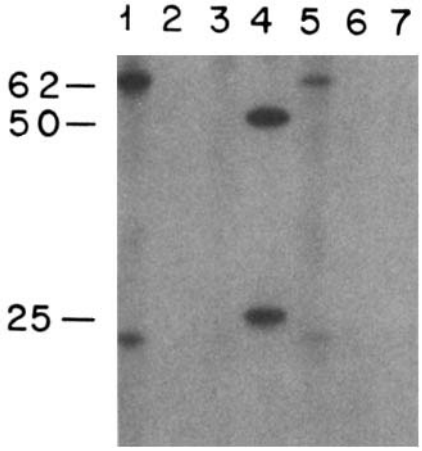

FIGURE 5. $BCL_1.2.58$ express sIgG that bears the BCL_1 idiotype. Cell lysates of radiolabeled $BCL_1.2.58$, $BCL_1.2.62$, and spleen cells were precleared with GAMμ-Sepharose and were then treated with RAMγ or $RABCL_1$ Id. The immune complexes were bound to GARIg-Sepharose and eluates of the Sepharose were analyzed by SDS-PAGE. Lanes *1* and *5* were cell lysates from $BCL_1.2.58$ cells, lanes *2* and *6* were cell lysates from $BCL_1.2.62$ cells, and lanes *3* and *7* were cell lysates from spleen cells. Lanes *1*, *2*, and *3* were precipitated with RAMγ; lanes *5*, *6*, and *7* were precipitated with $RABCL_1$ Id. Lane *4* contained iodinated MOPC-21 (IgG1) as molecular weight markers for γ and L chains. kD markers are shown.

(Fig. 5). It was found that under reducing conditions, the 62- and 25-kD peaks precipitated by RAMγ were only present in the $BCL_1.2.58$ cells (lane *1*), but not in the $BCL_1.2.62$ cells (lane *2*) or normal spleen cells (lane *3*). Furthermore, the sIgG could be immunoprecipitated with antiidiotype in $BCL_1.2.58$ (lane *5*). No IgG could be precipitated with $RABCL_1$ Id from lysates of $BCL_1.2.62$ cells (lane *6*) or spleen cells (lane *7*). These results indicate that the 62-kD species expressed on the $BCL_1.2.58$ cells is the γ chain of sIgG that expresses the BCL_1 idiotype.

To determine which subclass of IgG is expressed on the $BCL_1.2.58$ cells, the lysate of radioiodinated cells was treated with GAMμ-Sepharose and 200 μl of 10% *S. aureus*. The SN was then treated with RAML, RAMγ1, RAMγ2a, RAMγ2b, or RAMγ3 and then GARIg-Sepharose. It was found that *S. aureus* did not remove the 62- and 25-kD species, whereas RAML and RAMγ1 but not RAMγ2a, RAMγ2b and RAMγ3 subsequently precipitated sIgG (data not shown). Therefore, the $BCL_1.2.58$ cells express BCL_1 Id^+, sIgG1.

The Heavy Chain Variable Region Rearrangement is Identical in $BCL_1.B_1$ and $BCL_1.2.58$ Cells. Genomic DNAs isolated from BALB/c liver, $BCL_1.B_1$, $BCL_1.2.58$, and $BCL_1.2.62$ were digested with Eco RI and were probed with ^{32}P-labeled J_H3-J_H4-specific fragment. As shown in Fig. 6, this probe hybridized to the predicted 6.7-kb germline fragment of DNA from BALB/c liver (lane *1*). In contrast, the J_H3-J_H4 probe hybridized to a single 4.0-kb fragment in $BCL_1.B_1$ (lane *2*), $BCL_1.2.62$ (lane *3*), and $BCL_1.2.58$ (lane *4*) indicating that both clones use the same variable region rearrangement as that of the parental $BCL_1.B_1$ line. To exclude the possibility of identically migrating fragments, alternative enzyme digests (Xba I, Bam HI, Bgl I and Hind III) probed with J_H3–J_H4 were carried out. In all cases, parental and variant DNAs gave a single, identically migrating VDJ_{H2}-containing band (data not shown). The size of this fragment was the same

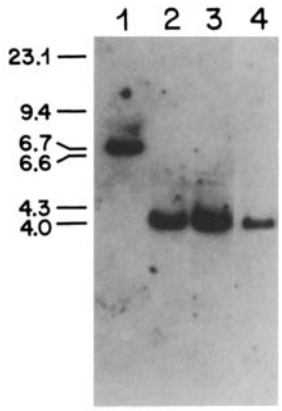

FIGURE 6. The J_H rearrangment is identical in $BCL_1.B_1$ and $BCL_1.2.58$. Genomic DNAs extracted from BALB/c liver, $BCL_1.B_1$, $BCL_1.2.62$, and $BCL_1.2.58$ were digested with Eco RI, were blotted as described in Materials and Methods, and were probed with ^{32}P-labeled J_H3J_H4 fragment. Lane *1*, BALB/c liver DNA; lane *2*, $BCL_1.B_1$; lane *3*, $BCL_1.2.62$; lane *4*, $BCL_1.2.58$. kb markers are shown in left margin.

as that predicted from the cloned BCL_1 variable region gene (reference 38, and P. Tucker, unpublished data). The Southern hybridization data, therefore, are consistent with those obtained from surface phenotyping and immunoprecipitation, and suggest that the IgG1 synthesized by the $BCL_1.2.58$ cells uses the same variable region rearrangement and idiotype as the BCL_1 IgM.

Northern Analysis of $BCL_1.2.58$ RNA. Total cellular RNA from $BCL_1.B_1$, $BCL_1.2.58$, and from various hybridomas was purified and analyzed by Northern blotting. When probed for μ (Fig. 7*a*), two hybridizing species were observed for both $BCL_1.B_1$ (lane *1*) and $BCL_1.2.58$ (lane *2*) at ~2.6 and 2.2 kb, which correspond to the μm (2.7 kb) and μs (2.4 kb) forms of the RNA (39). Conversely, when filters were probed with the γ1-specific probe (Fig. 7*b*), the membrane (3.5 kb) and secreted form (1.7 kb) of the γ1 mRNA were detected in the preparation of $BCL_1.2.58$ RNA (lane *2*), but not in the $BCL_1.B_1$ RNA (lane *1*). As in the case of μ mRNA, the γ1 mRNA in the $BCL_1.2.58$ cells migrated slightly faster than those in the control (lane *4*), most probably as a result of loading differences between lanes, since we have consistently observed that the 28S and 18S ribosomal RNA migrate further in the lanes with 15 μg of RNA than in those with 2.5 μg of RNA. In addition, the γ1 probe hybridized only to RNA from the IgG1 secreting hybridoma (lane *4*), thereby supporting the isotype assignment based on serology. The specificity of the γ1 probe was confirmed by rehybridizing the filters with γ2b and γ3-specific probes. These probes detected the appropriate control RNAs but did not hybridize to RNA in $BCL_1.B_1$ or the $BCL_1.2.58$ cells (data not shown).

Discussion

In the present report, we describe a variant of the $BCL_1.B_1$ tumor line which (*a*) contains mRNA for both the membrane and secreted forms of μ and γ1; (*b*) expresses sIgM and sIgG1 with the same idiotype; and (*c*) secretes IgM and IgG1. Thus, unlike the parent line, which expresses high levels of sIgM (and low levels

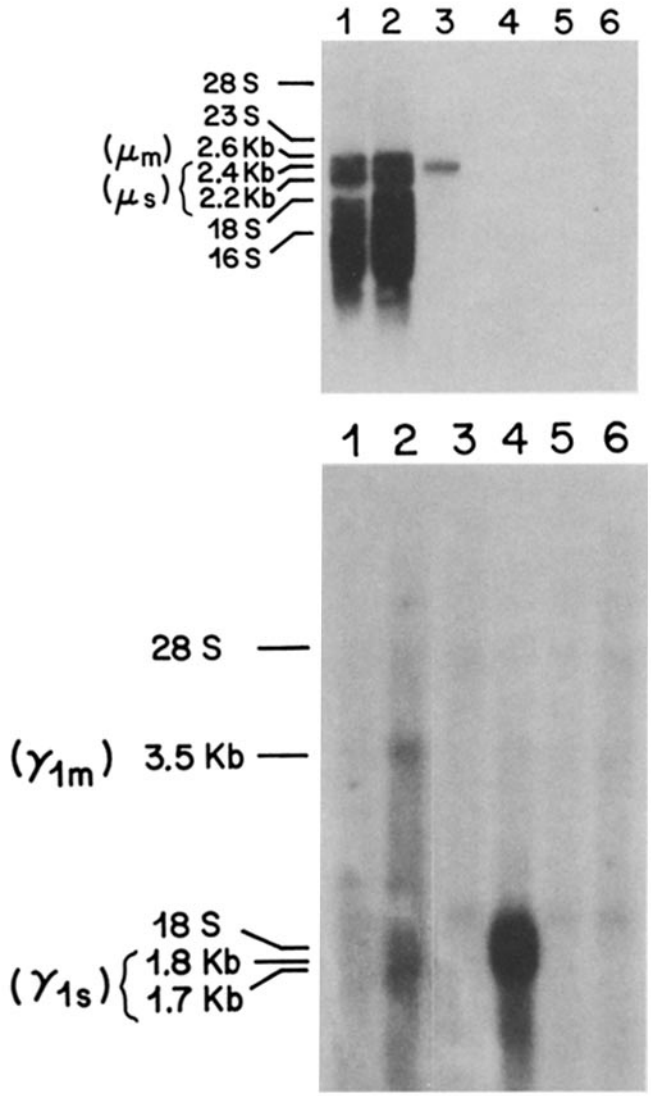

FIGURE 7. Analysis of steady-state levels of RNA in $BCL_1.B_1$ and $BCL_1.2.58$. 15 μg of RNAs isolated from $BCL_1.B_1$ cells, $BCL_1.2.58$ cells, and 2.5 μg RNAs from IgM-, IgG1-, IgG2b-, and IgG3-secreting hybridomas were electrophoresed and transferred to nitrocellulose filters. The RNA filters were probed with either (*a*) a μ-specific cDNA fragment or (*b*) a γ1-specific fragment. Lane *1*, $BCL_1.B_1$; lane *2*, $BCL_1.2.58$; lane *3*, IgM-secreting hybridoma; Lane *4*, IgG1-secreting hybridoma; lane *5*, IgG2b-secreting hybridoma; and lane *6*, IgG3-secreting hybridoma. μm denotes the membrane form of μ mRNA; μs denotes the secreted form of μ mRNA. Ribosomal RNA of eukaryotic (28S and 18S) and prokaryotic (16S and 23S) origin were included as markers. The smear of hybridization below the 18S marker in (*A*) appears to be μ-specific degradation.

of sIgD) and secretes only IgM, this variant line synthesizes, expresses, and secretes both IgM and IgG1.

The IgM and IgG1 synthesized by $BCL_1.2.58$ cells use the same idiotype, as evidenced by immunoprecipitation of the γ chain by the $RABCL_1$-Id antibody

that was generated by immunization of rabbits with BCL_1-IgM. The Southern hybridization data further support this notion, in that only a single VDJ_{H2}-containing fragment was detected when various digests of genomic DNA were probed with a J_H fragment. This implies that the same VDJ is being used for both μ and $\gamma 1$. However, proof that IgM and IgG1 of $BCL_1.2.58$ have an identical heavy chain variable region will require amino acid or cDNA sequencing of the μ and $\gamma 1$ chains.

Individual B cells are reported to express multiple isotypes other than IgD (13, 40–45). Rudders et al. (40) and Gordon et al. (44) reported that human B cell tumors express both IgM and IgG. However, these studies did not completely exclude absorption of IgG to the cell surface via Fc receptors. In our studies, the higher molecular weight membrane forms of μ and γ chains were shown unequivocally by radioiodination and immunoprecipitation. In addition, all the cells from $BCL_1.2.58$ secrete both IgM and IgG1. The simultaneous synthesis of IgM and IgG1 by the clonal population offers a unique model to examine the molecular mechanism(s) underlying this dual expression.

The coexpression of IgM and IgG1 on the cell surface, as well as the simultaneous secretion of both isotypes, deserves comment. The $BCL_1.2.58$ was selected based on secretion and was then found to express both sIgM and sIgG1. Switch variants isolated from hybridoma and myeloma cell lines were generally selected based on the expression of surface Ig (46, 47). These variants were also found to be secretion variants. In the studies of Thammana and Scharff (48), however, a hybridoma variant was isolated based on secretion. This variant was found to express IgG1 on the surface. It is possible that during normal B cell differentiation, switching from synthesis of IgM to synthesis of another isotype (other than IgD) involves expression of the new isotype on the cell surface, followed by its secretion. An example of this sequence may be the generation of IgG memory cells responsible for secondary antibody responsiveness.

Summary

We have subcloned the in vitro–adapted murine B cell leukemia, $BCL_1.B_1$, to obtain a variant that expresses both IgM and IgG1. By fluorescence analysis, radioiodination, and immunoprecipitation of cell surface Ig, and by RIA of medium from limiting dilution cultures, we have shown that: (*a*) all the cells express and secrete both isotypes. The heavy chains of both IgG1 and IgM have the apparent molecular weights of membrane μ and $\gamma 1$ chains; (*b*) both isotypes bear the same idiotype as determined by immunoprecipitation with antiidiotypic antibody, and both use the same VDJ rearrangement as shown by Southern blotting; and (*c*) the cells express the membrane and secreted forms of mRNA for both μ and $\gamma 1$ but not $\gamma 2b$ or $\gamma 3$. Taken together, the data suggest that all the cells are synthesizing, expressing on their surface, and secreting two isotypes that use the same VDJ rearrangement in the DNA and express the same serologically-defined idiotype. The molecular basis responsible for the production of the two isotypes in a single cell is the subject of the accompanying paper.

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References

1. Nossal, G. J. V., A. Szenberg, G. L. Ada, and C. M. Austin. 1964. Single cell studies on 19S antibody production. *J. Exp. Med.* 119:485.
2. Nossal, G. J. V., N. Warner, and H. Lewis. 1971. Incidence of cells simultaneously secreting IgM and IgG antibody to sheep erythrocytes. *Cell. Immunol.* 2:41.
3. Andersson, J., A. Coutinho, and F. Melchers. 1978. The switch from IgM to IgG secretion in single mitogen-stimulated B cell clones. *J. Exp. Med.* 147:1744.
4. Zan-Bar, I., S. Strober, and E. S. Vitetta. 1977. The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. I. Surface immunoglobulin isotypes on primed B cells in the spleens. *J. Exp. Med.* 145:1188.
5. Zan-Bar, I., E. S. Vitetta, and S. Strober. 1977. The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. II. Surface Ig isotypes on unprimed B cells in the spleens. *J. Exp. Med.* 145:1206.
6. Zan-Bar, I., E. S. Vitetta, and S. Strober. 1978. The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. IV. Expression of a single predominant isotype on primed and unprimed B cells. *J. Exp. Med.* 147:1374.
7. Yuan, D., E. S. Vitetta, and J. R. Kettman. 1977. Cell surface immunoglobulin. XX. Antibody responsiveness of subpopulations of B lymphocytes bearing different isotypes. *J. Exp. Med.* 145:1421.
8. Kincade, P. W., A. R. Lawton, D. E. Bockman, and M. D. Cooper. 1970. Suppression of immunoglobulin G synthesis as a result of antibody-mediated suppression of immunoglobulin M synthesis in chickens. *Proc. Natl. Acad. Sci. USA.* 67:1918.
9. Lawton, A. R., R. Asofsky, M. B. Hylton, and M. D. Cooper. 1972. Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to μ chain. *J. Exp. Med.* 135:277.
10. Gowans, J. L., and J. W. Uhr. 1966. The carriage of immunological memory by small lymphocytes in the rat. *J. Exp. Med.* 124:1017.
11. Honjo, T., and T. Kataoka. 1978. Organization of Ig heavy chain genes and allelic deletion model. *Proc. Natl. Acad. Sci. USA.* 75:2140.
12. Obata, M., T. Kataoka, S. Nakai, H. Yamagishi, N. Takahashi, Y. Yamawaki-Kataoka, T. Nikaido, A. Shimizu, and T. Honjo. 1981. Structure of a rearranged $\gamma 1$ chain gene and its implication to immunoglobulin class-switch mechanism. *Proc. Natl. Acad. Sci. USA.* 78:2437.
13. Yaoita, T., Y. Kumagai, K. Okumura, and T. Honjo. 1982. Expression of lymphocyte surface IgE does not require switch recombination. *Nature (Lond.).* 297:697.
14. Rabbitts, T. H. 1978. Evidence for splicing of interrupted immunoglobulin variable and constant region sequences in a nuclear RNA. *Nature (Lond.).* 275:291.
15. Wabl, M. R., G. B. Beck-Engeser, and P. Burrows. 1984. Allelic inclusion in the pre-B cell line 18-81. *Proc. Natl. Acad. Sci. USA.* 81:867.
16. Sablitzky, F., A. Radbruch, and K. Rajewsky. 1982. Spontaneous immunoglobulin class switching in myeloma and hybridoma cell lines differs from physiological class switching. *Immunol. Rev.* 67:59.
17. Knapp, M. R., P. P. Jones, S. J. Brack, E. S. Vitetta, S. Slavin, and S. Strober. 1979. Characterization of a spontaneous murine B cell leukemia (BCL_1). I. Cell surface expression of IgM, IgD, Ia and FcR. *J. Immunol.* 123:992.

18. Vitetta, E. S., D. Yuan, K. Krolick, P. Isakson, M. Knapp, S. Slavin, and S. Strober. 1979. Characterization of the spontaneous murine B cell leukemia (BCL_1). III. Evidence for monoclonality by using an anti-idiotype antibody. *J. Immunol.* 122:1649.
19. Gronowicz, E. S., C. A. Doss, F. D. Howard, D. C. Morrison, and S. Strober. 1980. An *in vitro* line of the B cell tumor BCL_1 can be activated by LPS to secrete IgM. *J. Immunol.* 125:976.
20. Robertson, S., J. R. Kettman, J. S. Miller, and M. Norgard. 1982. Murine monoclonal antibodies specific for virulent treponema pallidum (Nichols). *Infection Immunity.* 36:1076.
21. Robertson, S., C. F. Frisch, P. A. Gulig, J. R. Kettman, K. H. Johnson, and E. J. Hansen. 1982. Monoclonal antibodies directed against a cell surface-exposed outer membrane protein of haemophilus influenzae Type b. *Infection Immunity.* 36:80.
22. Brooks, K., D. Yuan, J. W. Uhr, P. H. Krammer, and E. S. Vitetta. 1983. Lymphokine-induced IgM secretion by clones of neoplastic B cells. *Nature (Lond.).* 302:825.
23. Isakson, P. C., E. Pure, E. S. Vitetta, and P. H. Krammer. 1982. T cell-derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J. Exp. Med.* 155:734.
24. Nisonoff, A., F. C. Wissler, L. N. Lipman, and D. L. Woernley. 1960 Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. *Arch. Biochem. Biophys.* 89:230.
25. Pure, E., P. C. Isakson, J. W. Kappler, P. Marrack, P. H. Krammer, and E. S. Vitetta. 1983. T cell-derived B cell growth and differentiation factors. Dichotomy between the responsiveness of B cells from adult and neonatal mice. *J. Exp. Med.* 157:600.
26. Vitetta, E. S., S. Baur, and J. W. Uhr. 1971. Cell surface immunoglobulin. II. Isolation and characterization of immunoglobulin from mouse splenic lymphocytes. *J. Exp. Med.* 134:242.
27. Blin, N., and D. W. Stafford. 1976. Isolation of high molecular weight DNA. *Nucleic Acids Res.* 3:2303.
28. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.
29. Chirgwin, J. M., A. E. Pozybyla, R. J. McDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294.
30. Maniatis, T., A. Jeffrey, and D. B. Kleid. 1975. Nucleotide sequence of the rightward operator of phage λ. *Proc. Natl. Acad. Sci. USA.* 72:1184.
31. Rogers, J., P. Clarke, and W. Salser. 1979. Sequence analysis of cloned cDNA encoding part of an immunoglobulin heavy chain. *Nucleic Acids Res.* 6:3305.
32. Tucker, P., K. B. Marcu, N. Newell, J. Richards, and F. R. Blattner. 1979. Sequence of the cloned gene for the constant region of murine γ2b immunoglobulin heavy chain. *Science (Wash. DC).* 206:1303.
33. Wels, T. A., C. J. Word, D. Rimm, G. P. Der-Balan, H. M. Martinez, P. W. Tucker, and F. W. Blattner. 1984. Structural analysis of the murine IgG_3 constant region gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2041.
34. Calame, K., J. Rogers, P. Early, M. Davis, D. Livant, R. Wall, and L. Hood. 1980. Mouse Cμ heavy chain in immunoglobulin gene segment contains three intervening sequences separating domains. *Nature (Lond.).* 284:452.
35. Rigby, P. W. T., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237.
36. Cooper, M. D., J. Kearney, and I. Scher. 1984. B lymphocytes. *In* Fundamental Immunology. W. E. Paul, editor. Raven Press, New York. 43.

37. Vitetta, E. S., K. Brooks, P. Isakson, J. Layton, E. Pure, and D. Yuan. 1984. B lymphocyte receptors. *In* Fundamental Immunology, W. E. Paul, editor. Raven Press, New York. 221.
38. Knapp, M., S. Strober, C.-P. Liu, P. W. Tucker, and F. R. Blattner. 1982. Simultaneous expression of Cμ and Cδ genes in a cloned B cell line. *Proc. Natl. Acad. Sci. USA.* 79:2996.
39. Early, P., J. Rogers, M. Davis, K. Calame, M. Bond, R. Wall, and L. Hood. 1980. Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. *Cell.* 20:313.
40. Rudders, R., and J. P. Howard. 1977. Pathways of human B lymphocyte differentiation. A clonal transition between IgM and IgG synthesis in a leukemic B lymphocyte. *J. Immunol.* 119:283.
41. Gathings, W. E., A. R. Lawton, and M. D. Cooper. 1977. Immunofluorescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. *Eur. J. Immunol.* 7:804.
42. Abney, E. R., M. D. Cooper, J. F. Kearney, A. R. Lawton, and R. M. E. Parkhouse. 1978. Sequential expression of immunoglobulin on developing mouse B lymphocytes. A systematic survey that suggests a model for the generation of immunoglobulin isotype diversity. *J. Immunol.* 120:2041.
43. Hsu, C. C. S. 1984. Coexpression of multiple immunoglobulin isotypes on human B lymphocytes. *Immunol. Commun.* 13:403.
44. Gordon, J., and J. L. Smith. 1980. Immunoglobulin synthesis by neoplastic cells: model of a clonal transition from IgM to IgG synthesis. *J. Clin. Pathol.* 33:539.
45. Perlmutter, A. P., and W. Gilbert. 1984. Antibodies of the secondary response can be expressed without switch recombination in normal mouse B cells. *Proc. Natl. Acad. Sci. USA.* 81:7189.
46. Neuberger, M. S., and K. Rajewsky. 1981. Switch from hapten-specific immunoglobulin M to immunoglobulin D secretion in a hybrid mouse cell line. *J. Immunol.* 78:1138.
47. Baumhackel, H., B. Liesegang, A. Radbruch, K. Rajewsky, and F. Sablitzky. 1982. Switch from NP-specific IgG_3 to IgG_1 in the mouse hybridoma cell line S24/63/63. *J. Immunol.* 128:1217.
48. Thammana, P., and M. D. Scharff. 1983. Immunoglobulin heavy chain class switch from IgM to IgG in a hybridoma. *Eur. J. Immunol.* 13:614.