Evidence for Somatic Selection of Natural Autoantibodies

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Summary

Natural autoantibodies are primarily immunoglobulin M (IgM) antibodies that bind to a variety
of self-antigens, including self-IgG. Accounting for a large proportion of the early B cell repertoire,
such polyspecific autoantibodies are speculated to contribute to the homeostasis and/or competence
of the primary humoral immune system. Recent studies indicate that the leukemia cells from
most patients with chronic lymphocytic leukemia (CLL) also express such IgM autoantibodies.
Similarly, the leukemia cells from many CLL patients react with murine monoclonal antibodies
(mAbs) specific for crossreactive idiotypes (CRIs) associated with human IgM autoantibodies.
In particular, leukemic cells frequently react with G6, a mAb specific for an Ig heavy chain (H
chain)-associated CRI, and/or with 17.109, a mAb that defines a κ light chain (L chain)-associated
CRI. Generated against IgM rheumatoid factor (RF) paraproteins, G6 and 17.109 each recognize
a major CRI that is present in many IgM RF paraproteins. Furthermore, over 90% of the IgM
paraproteins found to bear both H and L chain–associated CRIs also are found to have RF activity.
Molecular characterization of these CRIs demonstrates that each is a serologic marker for expression
of a highly conserved Ig V gene. As such, the frequent production of IgM polyspecific autoantibodies
in CLL simply may reflect the frequent use of such highly conserved autoantibody-encoding
Ig V genes with little or no somatic mutation. To test this hypothesis, we generated murine
transfectomas to pair the 17.109-reactive κ L chain of SMI, a 17.109/G6-reactive CLL population,
with the Ig H chain of SMI or other G6-reactive leukemia cells or tonsillar lymphocytes.
Cotransfection of vectors encoding the Ig H and L chains of SMI generated transfectomas that
produce IgM$_κ$ RF autoantibodies reactive with human IgG1 and IgG4. In contrast to
G6/17.109-reactive IgM$_κ$ RF Waldenstrom's paraproteins, the SMI IgM$_κ$ also reacts with several
other self-antigens, including myoglobin, actin, and ssDNA. However, cotransfection of the SMI
L chain with a vector encoding any one of 10 different G6-reactive Ig H chains generated
transfectomas that produce IgM$_κ$ antibodies without detectable polyspecific autoantibody
activity. These results indicate that polyspecific antiself-reactivity of G6/17.019-reactive Ig is
dependent on the somatically generated Ig third complementarity determining region. Collectively,
these studies imply that selection may be responsible for the frequent expression of polyspecific
autoantibodies in CLL and early B cell ontogeny.

The leukemia cells from many patients with chronic lymphocytic leukemia (CLL)[1] produce polyspecific IgM autoantibodies. Early studies by Preud'homme and Seligmann (1) indicated that CLL patients have leukemia cells that frequently bear surface membrane IgM that has RF activity, or binding activity for human IgG. In another limited survey, four of thirteen CLL patients were found to have leukemia cells that expressed surface IgM (sIgM) with binding activity

for human IgG (2). Subsequent studies demonstrated that over half of all CLL patients have leukemia cells that can be stimulated to secrete autoantibodies in vitro that bind to a variety of self-antigens, most notably human IgG (3–6). Although each of the leukemia cell cultures in these studies was monoclonal, the secreted autoantibodies generally were polyspecific, each Ig binding to two or more distinct self-antigens, e.g., human IgG, ssDNA, dsDNA, histones, cardiolipin, actin, thyroglobulin, and/or cytoskeletal components. Collectively, these studies indicate that the leukemia cells from most CLL patients may express polyreactive autoantibodies.

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[1] Abbreviations used in this paper: BBS, borate buffered saline; CLL, chronic lymphocytic leukemia; CRI, crossreactive idiotypes.

Similarly, the leukemia cells of many CLL patients react with mAbs specific for crossreactive idiotypes (CRIs) of IgM autoantibodies. Approximately one-fifth of the patients with κ L chain-expressing CLL, for example, were found to have leukemia cells that express a κ L chain-associated CRI defined by a mAb, designated 17.109 (7, 8). Furthermore, a high proportion of CLL patients have leukemia cells that react with G6 (8, 9), a mAb specific for an Ig H chain-associated CRI. Generated against monoclonal IgM RF paraproteins, both 17.109 and G6 recognize CRIs present on many IgM autoantibodies, particularly RFs (10–12). In addition, over 90% of the monoclonal IgM paraproteins that have both H and L chain CRIs are noted to have RF activity (13).

Evaluation of the molecular basis for expression of these CRI reveals that each is a serologic marker for a conserved Ig V gene expressed with little or no somatic mutation (14, 15). For example, the 17.109-reactive (17.109$^+$) neoplastic B cells from unrelated patients express nearly identical V_κ genes that share more than 99% nucleic acid sequence homology with a germline V_κIII gene isolated from placental DNA, designated *Humkv325* (14–16). Similarly, G6-reactive (G6$^+$) leukemia cells from unrelated patients, as well as G6$^+$ tonsillar lymphocytes from healthy donors (17), express homologous V_H genes of the V_H1 subgroup, designated 51p1 (18, 19). Conceivably, the frequent expression of IgM autoantibodies in CLL may be a direct consequence of the repeated use of such highly-conserved Ig V genes. This model is supported by the work of Sanz et al. (20), suggesting that natural polyreactive autoantibodies are encoded by a restricted repertoire of nonmutated Ig V genes.

However, this model minimizes the contribution of the third CDR (CDR3) to the autoreactive binding activity of the Ig expressed in CLL. This region of the Ig H chain is encoded by the D and J_H gene segments, that undergo recombination and NH_2-terminal nucleic acid base insertion immediately prior to V_H gene rearrangement (21, 22). Accordingly, the sequence of the CDR3 generally is idiosyncratic to each Ig V_H gene rearrangement, as has been noted with all G6$^+$ H chains sequenced to date (17, 18, and unpublished observations). In view of the large potential for diversity in the CDR3 of G6$^+$ H chains (17), the random pairing of a 17.109$^+$ κ L chain with any G6$^+$ Ig H chain may not be anticipated to form an autoantibody if the CDR3 is critical to autoantibody binding activity.

To examine this, we generated murine transfectomas to pair the 17.109$^+$ κ L chains of SMI, a 17.109/G6$^+$ CLL B cell population, with each of several different G6$^+$ H chains expressed by normal or leukemic B cells. We find that the myeloma cells cotransfected with the original pair of Ig H and L chain genes of SMI secrete polyreactive IgM$_\kappa$ RF autoantibodies. However, myeloma cells co-transfected with the SMI κ L chain gene and any one of ten different G6 H chain genes produced IgM$_\kappa$ that failed to have such polyspecific autoreactivity. Collectively, this study indicates that the polyspecific autoreactivity of the "natural" autoantibodies frequently noted in CLL and early B cell ontogeny is a selected specificity.

Materials and Methods

Antibodies. Purified polyclonal human IgG was obtained from Cappel Research Products (Durham, NC). We obtained purified myeloma proteins of each IgG isotype from Dr. Hans Spiegelberg (University of California, San Diego, School of Medicine, La Jolla, CA). Another set of purified myeloma proteins of each IgG isotype was purchased from Calbiochem Corp. (San Diego, CA). G6, a murine IgG1 mAb reactive with an autoantibody H chain-associated CRI (12) was obtained from Drs. Rizgar Mageed and Roy Jefferis (University of Birmingham, Birmingham, England). DA4-4, an IgG1 anti-human μ H chain-producing hybridoma (23), was obtained from the American Type Tissue Culture Collection (Rockville, MD). Anti-human κ or anti-human λ mAb-producing hybridomas were as described (7, 14). The IgM$_\kappa$ RF paraprotein BOR, isolated from the plasma of a patient with mixed cryoglobulinemia, was donated by Dr. R. Wistar, Jr. (National Naval Medical Center, Bethesda, MD). The IgM Waldenstrom's paraprotein, ME591, is as described (24). MOPC-21, an IgG1$_\kappa$ mouse myeloma protein of unknown specificity, was produced by P3X63Ag8 (25). This cell line was obtained from the American Type Culture Collection. Antibodies were precipitated from ascites or culture supernatants in 50% ammonium sulfate. After at least 1 h at 4°C, the samples were spun for 10 min at 10,000 g. The harvested Ig precipitate was suspended and dialyzed extensively in PBS, pH 7.2. Column chromatography of transfectoma Ig was performed with Superose-6™ in PBS at 4°C using an FPLC apparatus (Pharmacia, Uppsala, Sweden).

Vector Constructions. We isolated the rearranged gene complexes encoding the κ L chain and the μ H chain of G6-CRI$^+$/17.109-CRI$^+$ leukemic cells of CLL patient, SMI. For this we partially digested SMI genomic DNA with Sau3a. Large sized fragments were isolated on sucrose density gradients for ligation into λ dash™ with BamHI compatible overhangs (Stratagene Inc., La Jolla, CA), as described (26). For the κ L chain, a 17.8-kb BamHI fragment was isolated that contained the κ L chain promoter, rearranged V_κ gene, L chain enhancer and κ L chain constant region exon. This fragment was ligated in pSV2-gpt, a SV40 DNA plasmid vector with the *Escherichia coli* gpt gene (*Ecogpt*), encoding xanthine-guanine phosphoribosyltransferase (27). Expression of this gene allows for selection of transfected mammalian cells with mycophenolic acid (27, 28), as described (29) (see Fig. 1 *A*). In addition, a recombinant clone was isolated from the SMI genomic library that contained the rearranged H chain of SMI. An EcoRI/HindIII digest of this recombinant insert yielded a 3.4-kb fragment that contained the Ig promoter, the functionally rearranged V_H gene and the H chain enhancer region. This fragment was subcloned into Bluescript™ (Stratagene Inc.).

Unique restriction sites were introduced at sites flanking the H chain variable region gene to allow for the removal and subsequent insertion of different rearranged V_H genes isolated via PCR. For this, ssDNA was synthesized using the R408 helper phage (Stratagene Corp.). Unique restriction enzyme sites then were introduced via site-directed mutagenesis using synthetic oligonucleotides and the Oligonucleotide-Directed-Mutagenesis-System™ version 2 (Amersham Corp., Arlington Heights, IL). A ClaI site was introduced 5' of the leader sequence and a SalI site was introduced 3' of the J_H segment. Mutants initially were screened by digestion of plasmids prepared from independent colonies and checked by dsDNA sequencing using oligonucleotide complementary to sequences 100-bp contiguous to the introduced site, as described (14).

This modified 3.4-kb rearranged V_H gene fragment was ligated onto an 7.4-kb BamHI-HindIII fragment containing the human μ constant region exons excised from pN·x-μTNP (30), that originally was derived from the cloned segment λ C75 (31. This fragment, in turn, was ligated onto an EcoRI/BamHI fragment of pN·x-μTNP (30), containing the E. coli neo^r gene, the SV40 ori, pBR322 ori, and ampicillin resistance gene to create pRTM1 (see Fig. 1 B). The plasmid pN·x-μTNP originally was obtained from Dr. M. J. Shulman (University of Toronto, Toronto, Canada) (30).

Synthetic Oligonucleotides. PCR primers, site-directed mutagenesis oligonucleotides and probes were synthesized on a Pharmacia Gene Assembler (Pharmacia). Probes were 5' end-labeled with γ-[^{32}P]ATP and polynucleotide kinase, as described (7).

Cloning Rearranged G6-encoding V_H Genes Into pRTM1. Rearranged G6-encoding V_H1 genes isolated from normal tonsillar (17) or neoplastic B cells (18) were cloned and sequenced in Bluescript™ (Stratagene Inc.) as described (15, 17, 18). The rearranged V_H1 genes of SIC, HEN, BRA, and HUR were cloned from the genomic DNA isolated from CD5 B cell lymphoma tissues of patients with G6-reactive CLL (8). SMI, AND, and NEI were isolated from G6-reactive leukemia cells (18). To clone each rearranged V_H1 gene into pRTM1, we performed PCR on each recombinant plasmid using oligonucleotides specific for the V_H1 leader sequence (dGCA TCG ATA ATC ACC ATG GAC), or to a J_H consensus sequence (dGCG TCG ACT CTG AGG AGA CGG TGA) that have the flanking restriction enzyme sites ClaI or SalI, respectively. This generated PCR products of ∼660 bp that were digested with ClaI and SalI, to yield V_H1-containing inserts that were purified via agarose gel electrophoresis. These inserts were ligated into pRTM1 that had been digested with SalI and ClaI and then isolated free of the SMI V_H1 insert via 0.8% agarose gel electrophoresis. The ligated plasmids each were used to transform XL-1Blue™ (Stratagene, Inc.). Ampicillin-resistant transformants were screened via blot hybridization with a radiolabeled oligonucleotide (dGCG TAG TTT GCT GTA CC) specific for a sequence within the CDR2 of the V_H1 gene(s) encoding the G6-CRI, as described (18, 32).

Transfectomas. P3X63-Ag8.653, a nonsecreting mouse myeloma cell line (33), was obtained from the American Type Culture Collection. These cells were cultured in RPMI-1640 (Whittaker Bioproducts, Walkersville, MD) containing 20% FCS (HyClone Laboratories, Inc., Logan, UT) and 2-mM L-glutamine (Mediatech Inc., Washington, DC). Cells were washed and then suspended in ice cold RPMI at 1.5 × 10^7/ml. One ml of the cell suspension was then mixed with 2-mg linearized vector DNA and then transferred into a cold plastic cuvette containing two aluminum electrodes (Bio-Rad Laboratories, Richmond, CA). An electric pulse of 1,000 V/cm with a capacitance of 960 mF was applied to the solution using a Gene-Pulser™ apparatus (Bio-Rad Laboratories), as described (34). After the electric pulse, the cells were kept on ice for 10 min and then transferred to a flask of warm culture medium. 2 d after the electroporation, the medium was adjusted to 1 mg/ml of G418 (Geneticin; Gibco Bethesda Research Laboratories, Gaithersburg, MD) and/or 250 mg/ml xanthine (Sigma Chemical Co., St. Louis, MO), 15 mg/ml hypoxanthine (Sigma Chemical Co.), and 1 mg/ml mycophenolic acid (Sigma Chemical Co.), respectively, to select for transfectomas expressing E. coli neo^r and/or gpt genes of the transfected plasmids.

ELISA. To measure the concentrations of IgM, IgM$_\kappa$, or CRI-bearing Ig, polystyrene microtiter plates were incubated with murine mAbs HB-57 (anti–human IgM), 17.109, or G6, at 5–10 μg/ml of borate buffered saline (BBS, pH 8.2). After incubation for over 1 h, plates were washed and then exposed to 1% bovine

gelatin (J. T. Baker Inc., Phillipsburg, NJ) in BBS for 1 h at room temperature to block residual protein binding sites. Culture supernatants, concentrated transfectoma Ig, or purified IgM$_\kappa$ paraprotein were titrated in 1% gelatin in BBS and then added to the washed plates for a 1 h incubation at room temperature. Plates were then washed with 0.05% Tween in BBS before addition of alkaline phosphatase-conjugated goat antibodies specific for the human Ig μ chain or κ L chain (Southern Biotech., Birmingham, AL) to assay for IgM or IgM$_\kappa$, respectively. After another 1-h incubation at room temperature, plates were washed and then developed with aminoethylcarbozole in carbonate buffer (pH 9.8). The optical density at 405 nm of each of the wells was accessed using a ThermoMax™ (Molecular Devices Corp., Menlo Park, CA) ELISA plate reader linked to a MacIntosh^{Plus} (Apple Computer, Cupertino, CA) computer for protein concentration analyses using Δ SOFT™ software (BioMetallic Inc., Princeton, NJ). For standards we used the IgM$_\kappa$ RF paraprotein, BOR, and an IgM$_\kappa$ Waldenstrom's paraprotein, ME591, of irrelevant specificity. Binding to other self-antigens was measured similarly, except that the polystyrene plates were coated with bovine actin, bovine thyroglobulin, purified singlestranded calf thymus DNA (Sigma Chemical Co.), poly-deoxyinosine (poly dI), or poly-deoxythymidine (poly dT) (Pharmacia Inc., Piscataway, NJ). Plates without antigen but treated with 1% gelatin were used to measure gelatin binding activity. For this, test samples were diluted in 1% BSA faction V (Boehringer Mannheim Biochemicals, Indianapolis, IN), or in 0.05% gelatin without any detectable difference in plate binding activity.

To evaluate the ability of anti-idiotype to inhibit antigen binding, varying amounts of murine mAb were added to individual wells containing SMI IgM$_\kappa$ at a final concentration of 2 μg/ml. After a 30-min incubation at room temperature, the samples were assessed for their ability to bind antigen-coated plates, as described above. The binding activities of antibody with inhibitor were compared with those of serial dilutions of antibody without inhibitor to obtain the effective antigen-binding antibody concentrations. The percent inhibition of antigen binding was calculated as [1-(effective antigen-binding antibody concentration)/(actual concentration)] × 100.

Results

We generated transfectomas to pair different G6$^+$ IgM H chains with the 17.109$^+$ κ L chain of SMI, a 17.109/G6$^+$ CLL cell population. For this, we generated pRTM1, a human μ chain expression vector (Fig. 1 B). This vector contains the functionally rearranged SMI V_H1 gene flanked by unique restriction enzyme sites, ClaI and SalI, that were introduced via site-directed mutagenesis. This vector enabled us to exchange the SMI H chain variable with any one of several different functional V_HDJ_H exons of other G6$^+$ leukemia cells or tonsillar lymphocytes.

From pRTM1, we generated ten additional vector constructs that encode highly homologous Ig H chain variable regions (Fig. 2). Each variable region is encoded by a rearranged V_H1 gene that shares >99% nucleic acid sequence homology to 51p1, a V_H1 gene expressed during early fetal development (17–19, and unpublished observations). Like SMI, H chain constructs SIC, HEN, AND, NEI, BRA, and HUR were derived using the rearranged V_H1 gene expressed by G6$^+$
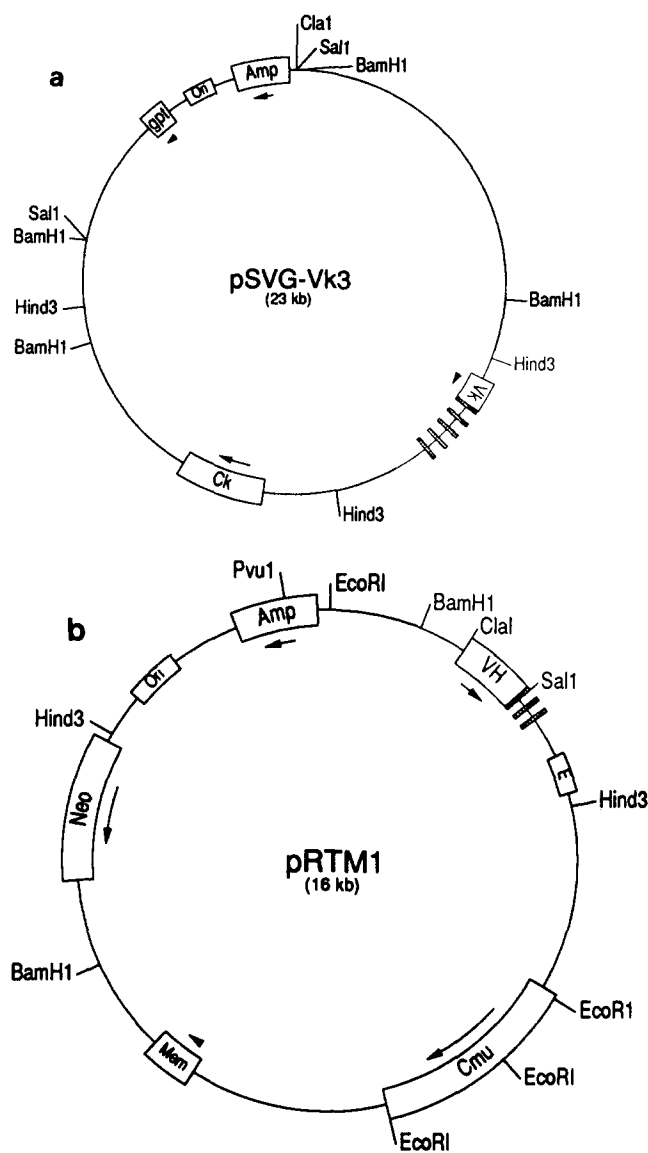
Figure 1. Structure of pSVG-Vk3 and pRTM1 plasmids. (←) Directions of gene transcription; (*open labeled boxes*) important regions. (*a*) pSVG-Vk3 contains the functionally rearranged Humkv325 V_κ gene and C_κ exon of SMI (17.8 kb) that was inserted into the BamHI site of pSVG-gpt (27, 28). V_κ, Humkv325 of SMI rearranged with J_κ1; C_κ, κ L chain constant region exon; gpt, *Ecogpt* and SV40 origin of replication: Ori, pBR322 origin of replication; Amp, ampicillin resistance gene. (*b*) pRTM1 contains the modified and functionally rearranged V_H gene of SMI and Ig H chain enhancer (3.4 kb) that was inserted between the EcoRI-HindIII sites of pN· x-μTNP (30), that previously flanked the functionally rearranged mouse V_HTNP gene. VH, the V_H1 gene expressed by SMI that is functionally rearranged to J_H4, and modified via site-directed mutagenesis to have unique flanking restriction enzyme sites ClaI and SalI; E, the Ig H chain enhancer region; $C\mu$, exons encoding the human C_μ constant region; Mem, C_μ membrane exon; Neo, the bacterial gene conferring neomycin resistance, the SV40 origin and SV40 early region; Ori, pBR322 origin of replication; Amp, ampicillin resistance gene.

CLL (8, 18, and unpublished observations). H chain constructs L26, L30, L33, and L42 were derived from the rearranged V_H1 genes expressed by G6$^+$ tonsillar B cells (17). From amino acid positions 1–94, L30 shares complete ho-

mology with SMI (Fig. 2). All other V_H1 genes have a GAA codon instead of an AAA codon at amino acid position 73, resulting in a lysine to glutamine substitution in the third framework region (Fig. 2). In addition, BRA has two other nonconservative nucleic acid base differences from SMI that result in serine to asparagine substitutions at amino acid positions 35 and 76. For comparison, the amino acid sequence of the H chain variable region of the G6$^+$ IgM$_\kappa$ RF paraprotein BOR (35) differs from SMI by nine residues (Fig. 2).

In contrast to the relative uniformity in the V_H gene-encoded protein sequences, the rearranged V_H genes differ markedly in the CDR3s (Fig. 2). In addition, the lengths of the CDR3s vary greatly, ranging from 6–25 codons (L26–SIC). Finally, rearranged J_H gene segments J_H3, J_H4, J_H5, and J_H6 are represented in this collection of G6$^+$ H chain variable regions (Fig. 2).

To generate transfectomas expressing each of these H chains with a 17.109$^+$ L chain, we generated pSVG-V$_\kappa$3. This vector contains the genomic DNA of the rearranged κ L chain gene complex of SMI that encodes a 17.109$^+$ L chain (Fig. 1 *A*). Two other G6$^+$ neoplastic B cell populations represented in this study, SIC and BRA, also originally expressed 17.109$^+$ κ L chains (15). In addition, the reference IgM$_\kappa$ RF paraprotein BOR also bears the 17.109 CRI (35). The deduced or actual protein sequences of these κ variable regions are highly homologous, reflecting expression of the conserved V$_\kappa$ gene, Humkv325, with little or no somatic mutation (Fig. 3) (7, 15, 16).

Cotransfection with pSVG-V$_\kappa$3 and the different constructs of pRTM1 generated transfectomas that each secreted intact human IgM$_\kappa$ protein. Sample concentrations were determined via ELISA using monoclonal IgM$_\kappa$ paraproteins, ME591 and/or BOR, as standards. The amounts of measured IgM$_\kappa$ protein in the supernatants varied between different transfectoma cell lines, ranging from a few hundred nanograms to several micrograms of antibody per ml. However, the Ig concentration of each supernatant assessed using plates coated with anti–human μ chain mAb was comparable to that determined using plates coated with G6, 17.109, or mAbs specific for human κ L chains (data not shown). Column chromatography of transfectoma proteins SMI, HEN, SIC, or L26 revealed the measured IgM$_\kappa$ or Ig κ activity to have a molecular size similar to that of IgG (data not shown). Collectively, these results indicate that each transfectoma apparently produces intact monomeric IgM$_\kappa$ molecules that bear both 17.109 and G6 CRIs.

Although each transfectoma secretes intact G6$^+$/17.109$^+$ IgM$_\kappa$, only the cell lines generated using the Ig H and L chain genes of SMI produced IgM$_\kappa$ with significant RF activity. Serial dilutions of concentrated IgM$_\kappa$ from each sample were assayed for binding activity to human IgG. The IgM$_\kappa$ produced by the transfectoma expressing both Ig chains of the SMI leukemia cell population (designated SMI) has detectable RF activity at concentrations below 1 μg/ml (Fig. 4). At any one IgM$_\kappa$ concentration, the RF activity of SMI is lower than that of the multimeric IgM$_\kappa$ RF paraprotein, BOR (Fig. 4). However, except for L30, none
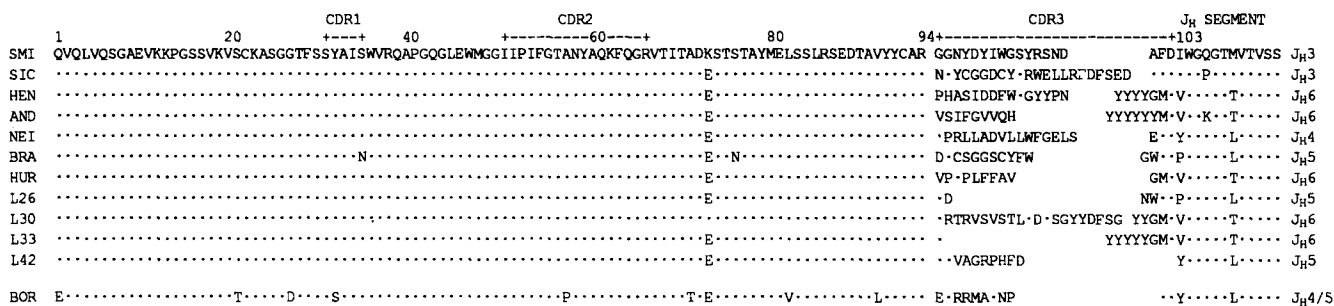
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                          CDR1                              CDR2                                                  CDR3              JH SEGMENT
         1          20    +---+    40      +--------60----+           80              94+--------------------------+103
SMI  QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYCAR GGNYDYIWGSYRSND          AFDIWGQGTMVTVSS  JH3
SIC  ·····················································································E······················· N·YCGGDCY·RWELLR:DFSED     ······P········  JH3
HEN  ·····················································································E······················· PHASIDDFW·GYYPN          YYYYGM·V·····T·····  JH6
AND  ·····················································································E······················· VSIFGVVQH                YYYYYYM·V··K··T·····  JH6
NEI  ·····················································································E······················· ·PRLLADVLLWFGELS         E··Y·····L·····  JH4
BRA  ·························N···························································E··N···················· D·CSGGSCYFW              GW··P·····L·····  JH5
HUR  ·····················································································E······················· VP·PLFFAV                GM·V·····T·····  JH6
L26  ·····················································································E······················· ·D                       NW··P·····L·····  JH5
L30  ·····················································································E······················· ·RTRVSVSTL·D·SGYYDFSG     YYGM·V·····T·····  JH6
L33  ·····················································································E······················· ·                        YYYYYGM·V·····T·····  JH6
L42  ·····················································································E······················· ··VAGRPHFD               Y·····L·····  JH5

BOR  E·················T·····D····S·······························P··············T·E······V·········L····· E·RRMA·NP               ··Y·····L·····  JH4/5
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Figure 2. Sequence comparison of the deduced or actual amino acid sequences of G6[+] Ig H chain variable regions. (*Right*) Code name for each sequence. The 20-letter amino acid code represents the deduced sequence of SMI. All other sequences are compared with that of SMI. (·) Homology between the compared sequences and SMI. Otherwise, letters correspond to the discordant amino acids. Spaces are introduced to maximize sequence homology in CDR3. Listed above SMI, amino acid residues are numbered according to Kabat et al. (51). Also listed are labels indicating the first, second, and third CDRs, and the region encoded by the J$_H$ gene segment. (*left*) Deduced J$_H$ gene segments used to encode the fourth framework of the H chain variable regions. At the bottom of the figure is the actual amino acid sequence of BOR (35) compared to that of SMI.
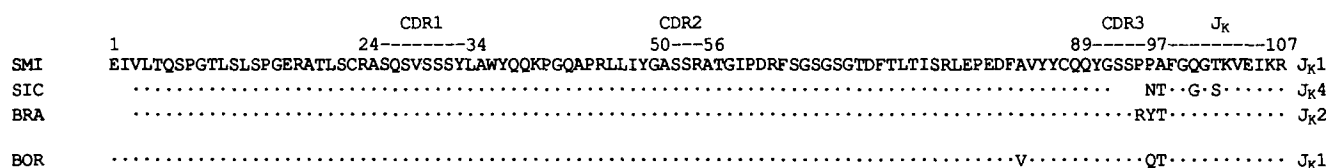
```
                                CDR1                  CDR2                                        CDR3        JK
         1               24-------34      50---56                                      89-----97----------107
SMI  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPPAFGQGTKVEIKR  JK1
SIC  ···········································································································NT··G·S······  JK4
BRA  ···································································································································RYT···········  JK2

BOR  ·······················································································································V···········QT···········  JK1
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Figure 3. Sequence comparison of the deduced or actual amino acid sequences of 17.109[+] Ig L chain variable regions. The amino acid sequences are described, and compared to SMI as in Fig. 2

of the other transfectomas IgM$_\kappa$ proteins had any detectable RF activity, even at their highest concentrations (>2 μg/ml for all except for AND [0.6 μg/ml]) (Fig. 4). Furthermore, although L30 had slight reactivity with human IgG, its RF activity only was detectable at concentrations exceeding 1 μg/ml (Fig. 4).

The SMI IgM$_\kappa$ RF is isotype specific. SMI has binding activity for IgG1 or IgG4 myeloma proteins that is similar to that for total human IgG (Fig. 5). In contrast, SMI binds poorly to IgG2 and IgG3. The same hierarchy of binding activity for IgG1 > IgG4 >> IgG2 > IgG3, is noted using either of two sets of purified myeloma proteins representing each of the IgG isotypes. As such, the reactivity of the SMI IgM$_\kappa$ for IgG1 or IgG4 cannot be ascribed to an idiosyn-

cratic binding activity for a particular myeloma protein of a single donor. Of note, the BOR IgM$_\kappa$ RF paraprotein also reacts primarily with IgG of the IgG1 isotype (data not shown).

In contrast to BOR and each of the other transfectoma proteins, the SMI Ig also has polyspecific antigen binding activity. The SMI IgM$_\kappa$ reacts strongly with myoglobin, actin, or ssDNA, and, by comparison, weakly with thyroglobulin (Fig. 6). That the ssDNA binding activity cannot be ascribed to trace residual chromatin is demonstrated by SMI's reactivity with synthetic polydeoxyinosine (poly dI) or polydeoxythymidine (poly dT) (Fig. 6). In every case, the binding activity of SMI for each of these antigens is detectable at IgM$_\kappa$ concentrations below 1 μg/ml. In contrast, none of the other transfectomas IgM$_\kappa$ proteins, including L30, had detectable binding activity to any of these antigens, even
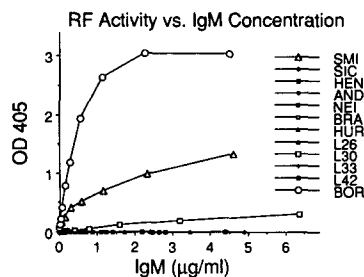

Figure 4. RF activity of each transfectoma protein compared with BOR. Ordinate is OD at 405 nm of the ELISA for RF activity measured on plates coated with total human IgG. The abscissa describes the IgM$_\kappa$ concentration (μg/ml) of the assayed sample. Symbols representing titration of each sample are indicated. In particular, (O) BOR, (△) SMI, and (□) binding activity of L30.
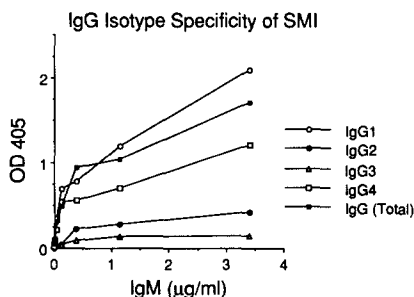

Figure 5. IgG isotype specificity of SMI. Graph labeled as in Fig. 4. Symbols represent titration of SMI on plates coated with IgG1 (O), IgG2 (●), IgG3 (△), IgG4 (□), or total IgG (■), respectively.
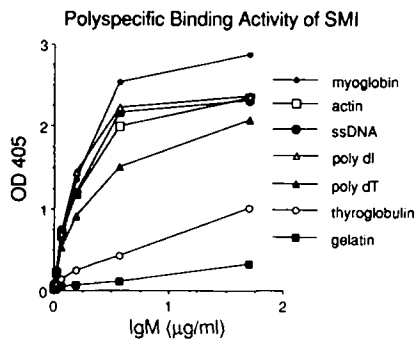
Figure 6. Polyspecific binding activity of SMI. Graph labeled as in Fig. 4. Symbols represent titration of SMI on plates coated with myoglobin (●), actin (□), single-stranded DNA (●), poly deoxyinosine (△), poly deoxythymidine (▲), thyroglobulin (O), or gelatin (■), respectively.
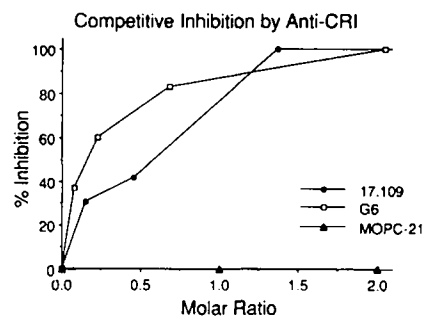

Figure 8. Inhibition of SMI antigen binding activity by anti-CRI mAb. Ordinate lists percent inhibition of SMI binding to myoglobin-coated plates by preincubation of SMI with either 17.109 (●), G6 (□), or MOPC-21 (△), calculated as described in Materials and Methods. The measured values are plotted against the molar ratio of anti-idiotypic or MOPC-21 inhibitor to SMI IgM$_\kappa$.

at the highest tested concentration of >2 μg/ml for all except AND (1.6 μg/ml) (data not shown). In addition, the IgM$_\kappa$ RF, BOR, also did not have any detectable binding activity for any of these antigens, even at concentrations in excess of 4 μg/ml. None of the transfectoma proteins, including SMI, had significant binding activity for bovine gelatin (data not shown, and Fig. 6).

We size fractionated the SMI IgM$_\kappa$ via FPLC and evaluated each fraction for antigen binding activity by ELISA. These analyses revealed that the predominant IgM actin-binding activity of SMI is similar in size to monomeric IgG (Fig. 7). Similarly, the IgM antimyoglobin binding activity of SMI also comigrated with human IgG (data not shown). These results demonstrate that the observed polyspecific antigen-binding activity of SMI is not dependent on a multimeric structure.

Finally, the polyspecific binding activity of SMI could be inhibited specifically by anti-CRI mAbs. Varying amounts of IgG murine mAbs were added to individual wells containing SMI IgM$_\kappa$ at a final concentration of 2 μg/ml. After a 30-min incubation at room temperature, the samples were assessed for their ability to bind myoglobin via ELISA. The

Figure 7. Size chromatography of SMI antigen binding activity. The SMI IgM$_\kappa$ was size fractionated via FPLC. Each fraction was assayed for human IgM anti-actin activity by ELISA. The ELISA OD at 405 of SMI (O) is plotted against the fraction sample number. Also shown are ODs at 280 nm of the sample fractions of ME591, a pentameric Waldenstrom's IgM$_\kappa$ paraprotein (□), or monomeric IgG (△).

percent inhibition of antigen binding were plotted versus the molar ratios of murine IgG inhibitor to SMI IgM. We find that either 17.109 or G6 can completely inhibit SMI antigen binding activity at molar ratios in slight excess of unity (Fig. 8). In contrast, MOPC-21, a control mouse myeloma IgG1 protein of unknown specificity, did not inhibit SMI antigen binding activity (Fig. 8).

Discussion

This study demonstrates that the random pairing of different G6$^+$ Ig H chains with a single 17.109$^+$ κ L chain generally does not produce an IgM autoantibody. Of 11 murine transfectomas that express IgM$_\kappa$ proteins bearing both the G6 and 17.109 CRIs, only the native Ig H and L chain pair had significant autoantibody activity. This cannot be attributed to defective Ig synthesis by the other transfectomas. The culture supernatants of each transfectoma were found to have stoichiometric concentrations of Ig μ chains and κ L chains by ELISA. Furthermore, ELISA plates coated with either anti-κ L chain antibodies or anti-CRI mAbs captured amounts of transfectoma IgM that were similar to those of anti-hu-IgM-coated plates. Finally, column-chromatography of representative samples of the IgM antibodies from those with or without RF activity indicated that these proteins have a molecular size similar to that of monomeric IgG. Collectively, these results indicate that each transfectoma produces intact monomeric human IgM$_\kappa$ molecules bearing both 17.109 and G6 CRIs. We conclude that the random pairing of different G6$^+$ Ig H chains with 17.109$^+$ κ L chains generally does not generate an Ig with antiself reactivity.

The H chain constant regions of the transfectoma IgM proteins examined in this study are encoded by human C$_\mu$ exons derived from an IgM expression vector, designated pN· x-μTNP. In previous studies, Boulianne et al. noted that Sp2/0 cells cotransfected with pN· x-μTNP and an Ig L chain expression vector secreted mostly multimeric IgM (30). However, as noted above, we found that most of the IgM secreted by each transfectoma comigrates with monomeric human IgG
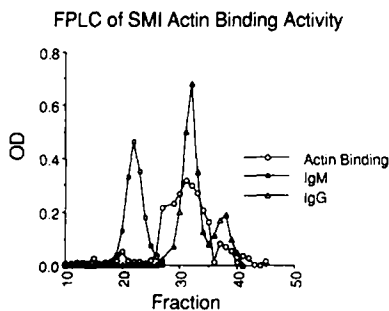
in FPLC. Conceivably, cell culture conditions and/or processing procedures may have influenced the ratio of IgM monomers to IgM multimers that is secreted by our transfectomas (Dr. Marc J. Shulman, personal communication). Alternatively, a mutation(s) may have occurred in the human C_μ exons during the construction of pRTM1, resulting in a vector that encodes Ig μ chains that are inefficient in forming multimers (36, 37). Restriction mapping studies of pRTM1 and pN·x-μTNP, however, failed to reveal any differences between the two vectors in the regions containing the Ig C_μ exons (data not shown). In any case, all transfectomas used in this study possessed the same Ig C_μ exons, were cultured under similar conditions and apparently secreted mostly monomeric IgM. Furthermore, most of IgM autoantibody activity of SMI also chromatographically comigrates with human IgG (Fig. 7). Therefore, the differences in the binding activities of the transfectoma proteins bearing both 17.109 and G6 CRIs cannot be ascribed to simple differences in Ig receptor valency.

The V_H1 exon of SMI differs from those of most other transfectomas by a single nonconservative base substitution at amino acid position 73, resulting in a glutamine to lysine substitution (Fig. 2). Although this substitution may be due to somatic mutation, this difference also may reflect a genetic polymorphism in the V_H1 gene encoding the G6-CRI. Possibly, the lysine residue at position 73 contributes to the RF binding activity of the SMI transfectoma protein. In this regard, it should be noted that L30, the only other transfectoma IgM with lysine at amino acid position 73 (Fig. 2), also is the only other transfectoma protein that has binding activity for IgG that is above background (Fig. 4). It is not likely that this substitution enhances the RF activity of G6-reactive IgM proteins, in general, however, as most other G6-reactive RF autoantibodies and CLL Ig do not have a lysine at this position (e.g., BOR, Fig. 2, and references 12, 13, 35). Another possibility is that the CDR3 of the SMI L chain may have been selected for its ability to generate an RF Ig when paired with a G6-reactive H chain that has a lysine at this position. Evaluation of the relative contribution of this lysine residue to the RF activity of SMI may require site-directed mutagenesis studies and/or further chain mixing experiments.

In any case, the present study delineates the importance of the CDR3 to the polyspecific binding activity of Ig bearing these autoantibody-associated CRIs. Each of the G6$^+$ H chains has a unique CDR3 sequence. However, except for the lysine to glutamine substitution at position 73, the V_H gene-encoded protein sequences for all but one of the transfectoma proteins are identical. Moreover, L30 shares comparable homology with SMI in the V_H gene-encoded protein sequence. Since the pairing of L30 with the 17.109$^+$ SMI L chain does not produce an Ig with detectable polyspecific autoantibody reactivity, this amino acid substitution alone cannot account for the absence of such binding activity in the other transfectoma proteins. Collectively, these data argue that the somatically generated CDR3 may contribute significantly to the polyspecific autoantibody activity of certain G6$^+$/ 17.109$^+$ IgM proteins.

This indicates that such polyspecific binding activity of natural autoantibodies may be a selected specificity. In the primary follicles of human fetal spleen, high proportions of B cells express autoantibody-associated CRIs, such as G6 and 17.109 (38). Genetic mechanisms may favor the frequent rearrangement of Ig V genes that have the capacity to encode CRI-bearing IgM autoantibodies (39). However, as with our transfectoma studies, the random pairing of such Ig L and H chains during ontogeny may infrequently give rise to Ig with polyspecific antiself-reactivity. Conceivably, B cells that chance express functional and self-reactive receptors may be stimulated to mature into functional B lymphocytes. Such selection may contribute to the noted high frequency of B cell clones expressing such polyreactive natural autoantibodies during early development (40, 41). Indeed, recent data indicate that most peripheral B cells in mice may be ligand selected (42). The selection for self-reactive autoantibody-expressing B cells during B cell ontogeny may be similar to that of T cells in the thymus, where only the cells expressing receptors with low affinity antiself-reactivity are selected for subsequent T cell maturation (43-47).

With regard to CLL, it is not certain whether the apparent selection for polyreactive autoreactivity merely reflects its cytogenesis, or if it actually plays a role in leukemogenesis (48, 49). Recent studies indicate that most patients with CLL have leukemia cells that express IgM autoantibodies that, like the SMI transfectoma IgM$_\kappa$, react with a variety of different self antigens (3-6). The frequency of CLL patients that have leukemia B cells that express such polyreactive autoantibodies apparently greatly exceeds the noted frequency of polyreactive B cells in normal embryonic tissues, cord blood or adult peripheral blood. Whatever the origin, Guigou et al. (50) recently noted that 11-16% of the EBV-transformed Ig-secreting B cell clones expressed polyspecific antibodies when tested on a panel of nine antigens, including self-antigens. Conceivably, B cells selected for expression of sIg with autoantibody activity during early B cell ontogeny could be stimulated constitutively by self-antigen, thus increasing the risk for malignant transformation to CLL.

Finally, chance somatic mutation in the V genes encoding such self-reactive antibodies may enhance the affinity of the expressed Ig for self-antigen, contributing to augmented cell stimulation and B cell maturation. Unlike CLL, the neoplastic cells in patients with Waldenstrom's macroglobulinemia and/or mixed cryoglobulinemia have differentiated into Ig secreting cells. The IgM$_\kappa$ RF paraprotein BOR was isolated from the plasma of one such patient. It is important to note that the Ig H chain of the RF paraprotein BOR differs from the deduced amino sequence of 51P1 or SMI by several residues (35). Conceivably, these differences are secondary to somatic mutations that result in loss of polyspecific autoreactivity and higher affinity for the driving self-antigen(s), possibly human IgG. Further comparative studies on the structure-function relationships of such IgM autoantibodies may help elucidate factors involved in the etiopathogenesis of these lymphoproliferative diseases.

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