

Defective Secretion of an Immunoglobulin Caused by Mutations in the Heavy Chain Complementarity Determining Region 2

By Ching Chen, Tammy M. Martin, Susan Stevens,
and Marvin B. Rittenberg

*From the Department of Microbiology and Immunology, Oregon Health Sciences University,
Portland, Oregon 97201*

Summary

We have investigated four secretion-deficient antibodies (Abs) derived from a panel of 46 mutant T15 anti-phosphocholine Abs, all of which have point mutations in the heavy chain (H) complementarity determining region 2 (CDR2). The level of secretion for these four Abs was <10% of wild type when expressed together with the T15 light chain (L) in either SP2/0 or P3X63Ag8.653 myeloma cells although normal levels of H and L chain mRNA were produced. Moreover, abundant intracellular H and L chain proteins were detected. Three of the four mutants had little or no assembled H and L complexes intracellularly whereas one had a significant amount of intracellular immunoglobulin (Ig) which was shown to be capable of binding Ag. Thus, we demonstrate for the first time that point mutations confined to CDR2 of the H chain variable (V) region can impede Ab assembly and secretion. We then introduced the same CDR2 mutations into a related H chain which is encoded by the same T15 VH gene but different diversity (D) and joining (J) genes. When these H chains were expressed with a non-T15 L chain, the resulting Abs were secreted normally. The results thus suggest that the effects of the CDR2 mutations on Ab secretion are dependent on their interactions with L and/or H chain D-J sequences. These results also reveal a novel mechanism that could contribute to B cell wastage.

The process of somatic mutation appears to operate randomly throughout Ab V regions, however, because of the selective force of Ag, specific mutations that confer a binding advantage to the B cell become dominant and thus provide a biased view of the structural consequences of somatic mutations. Mutations that fail to improve Ag binding are less well understood since such Abs are not easily recovered, however, this is the category of mutation that is likely to contribute to B cell wastage during an Ag-driven response. We have chosen to analyze the structural consequences of V region mutations that result in loss of Ab function. Because formation of a functional Ab molecule is a complex, multi-step process, a number of mechanisms could contribute to loss of function, including those affecting interactions with Ag, protein assembly, and Ab secretion.

The pathways of Ig assembly were delineated in classic studies by Scharff and his colleagues (1, 2) who showed that the assembly of H and L chains of each isotype followed a major and a minor pathway. For IgG2b, the major pathway was $H+L \rightarrow HL \rightarrow H_2L_2$. The minor pathway consisted of $H+L \rightarrow HL \rightarrow HL+H \rightarrow H_2L+L \rightarrow H_2L_2$. The structure of the H chain isotype appeared to determine the pathway of assembly since most of the tumor cells studied utilized κ chains (1). Furthermore, it has been suggested based on H+L chain

reassociations in vitro, somatically mutated H chains may be more restricted in their ability to pair with heterologous L chains than unmutated H chains (3). Thus, mutations in either chain that affect assembly have the potential to prevent proper transport and secretion.

Studies of faulty Ab assembly and secretion have revealed several abnormal Ig products: secreted L chain monomers and dimers (4), secreted mutant H chains (5, 6), and secreted partially folded Ig (7). In most of these mutants the nonsecreted Ab is retained in the endoplasmic reticulum (ER)¹ (8). Free unmutated L chains can be secreted in the absence of H chains (9, 10), but free unmutated H chains are retained in the cell in the absence of L chain (11, 12). Mutant L chains that cannot be secreted have been found in the lumen of the ER (10, 13). Mutations at Gly 15 in V λ 2 (14) and Phe 62 in V λ 1 (15) have been shown to block secretion. Phe 62 \rightarrow Ser blocked transport of λ 1 from ER to the Golgi and prevented secretion even in the presence of H chain. The mutation at residue 62 did not cause gross misfolding since the mutants contained intracellular IgM that could bind Ag. More recently,

¹ Abbreviations used in this paper: ER, endoplasmic reticulum; PC, phosphocholine; WT, wild type.

in another H chain loss variant, His 87 in the MOPC 21 κ chain caused the latter's retention in the ER (16). Most L chains contain Tyr or Phe at position 87, and changing His 87 to Phe in V κ 21 restored secretion.

The location of these L chain mutations led to the proposal that VL contains information essential for Ig secretion (15) in accord with earlier reports that transfected L chains can restore Ig secretion in cells expressing only H chains (17–19). It is interesting to note that defective secretion of the MOPC21 κ chain (containing His 87) can be "repaired" by coexpression with H chain (16) as can defective secretion of another L chain, CH12 κ (20). Thus, some L chain secretion failures can be overridden by H chain. It was suggested that VH-VL assembly masks the negative influence of His 87, thereby allowing the protein to continue on the normal transport pathway. However, the molecular nature of the interaction is unclear, and it is important here not to rule out the possibility of a positive transport signal resident on H chains, as has been suggested previously (7).

Analysis of the H chain contribution to Ig assembly and secretion has focused on two domains of the constant region: CH1 and the μ s tailpiece. L chain-negative cell lines can secrete γ H chains in which CH1 is absent (7, 21, 22) and camels normally secrete IgG2 and IgG3 H chain dimers lacking CH1 and L chains (23). However in order for free μ chains to be secreted, it appears that CH1 must be deleted and a site (Cys 575) in the μ s tailpiece must be modified (6). These studies suggest that retention sites in the H chain constant region may be masked by L chain association or by polymerization into IgM pentamers before Ig transport proceeds.

We show here for the first time that mutations in the VH CDR2 region can also result in loss of Ab secretion. We have created four mutants of the anti-phosphocholine Ab, T15, that only differ by two to four point mutations in the VH CDR2 yet their secretion is <10% of wild type (WT). This is the first evidence that the VH hypervariable region may be involved in the process of Ab secretion. In addition, these results reveal a novel mechanism through which somatic mutation could contribute to the process of B cell wastage.

Materials and Methods

Genomic Cloning of D16 VH Genes. Genomic DNA obtained from D16 hybridoma cells was partially digested with EcoRI and ligated to λ phage EMBL4 EcoRI arms (Stratagene, La Jolla, CA). Approximately 10^6 phage recombinants were screened with JH- and VH1-specific probes (24). A clone positive for both probes was isolated and characterized by restriction enzyme analysis. The 7.1-kb EcoRI fragment containing the productive V μ 1-D μ SP2.2-J μ 2 rearrangement of D16 was subcloned into the pTZ18U phagemid (Bio-Rad Laboratories, Richmond, CA) for sequencing and subsequent genetic manipulation.

Plasmid Constructs. The CDR2 of the germline S107 VH1 gene was randomly mutated as described previously (25) and the WT and mutated T15 VH fragments were cloned into the pSV2gpt vector provided by Dr. J. Sharon (Boston University, Boston, MA) which contains the γ 2b C region gene (26). The pSV2neoV κ 22C κ plasmid, a gift from Dr. S. Morrison (University of California at Los Angeles, Los Angeles, CA), was described elsewhere (27). The

germline CDR2 sequence of the D16 VH1 gene was replaced with mutated VH1 CDR2 sequences from the T15 mutants generated previously (25) using SmaI and XhoI digestion and subsequent ligation into pTZ18U. The CDR2-replaced D16 VH genes were then subcloned into the pSV2gpt γ 2b plasmid. The VH1 sequences and the CDR2 mutations were verified by sequencing in both pTZ18U and pSV2gpt vectors using primers for VH1 framework 3 (25) and JH2 (24).

Cell Culture and Transfection. Cells were cultured in IMDM (GIBCO BRL, Gaithersburg, MD) containing l-glutamine, nonessential amino acids, sodium pyruvate, and 15% FCS. Mutant and WT T15 cell lines were described previously (25). The D16 hybridoma was produced and characterized in this laboratory by Dr. M. Stenzel-Poore. It was derived from an early secondary response to phosphocholine (PC)-KLH, in which a mouse received a primary injection of 100 μ g PC-KLH in CFA and was boosted on day 14 with 100 μ g PC-KLH in IFA. Spleen cells were fused with the myeloma cell line FO as described (28). D16 produces an IgM, V κ 1c, group II anti-PC-protein Ab. Accordingly, it binds nitrophenylphosphocholine much better than PC, is T15 id⁻ and does not bind to PC-polysaccharide (29). D16 is encoded by the S107 VH1 germline sequence and thus is identical to T15 in this respect. It differs from T15 in DJ and V κ . An H chain loss variant of D16 was isolated by soft agar cloning (30) using a mouse embryonic fibroblast feeder layer (C₃H/10T1/2, kindly provided by Dr. M. Wilkinson (Oregon Health Sciences University)).

SP2/0 myeloma, P3X63Ag8.653 myeloma, and H chain-loss D16 hybridoma cells were used as recipients for DNA transfection. Transfection was performed by the lipofectin method as described previously (25) except that 100 μ l lipofectin and a 22-h incubation was used in each transfection in the case of D16 cells. In this study, the SP2/0 cells were first transfected with a pSV2neoV κ 22C κ plasmid and a stable V κ 22C κ transfectant was then used as the recipient for subsequent transfection with WT or mutant VHS107- γ 2b. In the case of P3X63Ag8.653 cells, cotransfection of V κ 22C κ and VHS107- γ 2b was performed.

ELISA. The basic procedures of ELISA were described previously (31). Antibody concentration was determined by sandwich ELISA in which microtiter plates were coated with rabbit anti-mouse γ 2b (Zymed Laboratories Inc., South San Francisco, CA) or with goat anti-mouse κ (Southern Biotechnology Associates, Birmingham, AL). Serial dilutions of culture supernatant or cell lysate were added to the plates and alkaline phosphatase-labeled rabbit anti-mouse γ 2b or goat anti-mouse κ were used to detect the Ab. Purified Ab from hybridoma PCG2b-2 (γ 2b, κ) was used to generate standard curves. For T15 idiotype expression, three anti-idiotype reagents were used. The mAb B36-82 (32) and the goat anti-T15 antiserum are specific for T15 VH and VL regions and were described previously (25). The mAb TC54 was a kind gift from Dr. M. Scharff (Albert Einstein College of Medicine, Bronx, NY) and is specific for the S107 VH region (33). Purified anti-idiotypic Ab was coated on the plates and then overlaid with protein A-Sepharose-purified mutant Ab at 100 ng/ml. Binding was detected with alkaline phosphatase-labeled rabbit anti-mouse γ 2b.

Preparation of Cell Lysates. Cell lysates were prepared according to George et al. (34). Briefly, cell pellets were lysed in ice cold PBS containing 25 mM iodoacetamide, 20 μ g/ml soybean trypsin inhibitor, 50 μ g/ml PMSF, and 0.25% NP-40 (all from Sigma Chemical Co., St. Louis, MO). Lysates were incubated for 30 min at 4°C before spinning out nuclei and insolubles. For ELISA, cells were plated in duplicate 4-ml cultures at 2×10^6 cells/ml, incubated for 4 h at 37°C, washed twice in culture medium without

FCS, counted, and pelleted. The pellets were lysed in 100 μ l lysing solution and assayed immediately. For Western blotting, confluent 25 cm³ flasks of cells were lysed in a volume of lysing solution to give an equivalent of 2×10^8 cells/ml.

Western Blot Analysis. Cell lysates were run on a 12% SDS-PAGE gel under reducing conditions. The separated proteins were then electrophoretically transferred to an Immobilon P membrane (Millipore, Marlborough, MA). The blot was blocked overnight at 4°C in PBSA (PBS with 0.02% sodium azide) containing 3% nonfat dry milk (NFDm). The blot was incubated for 1.5 h at room temperature in rabbit anti-mouse IgG2b (Zymed) diluted to 1 μ g/ml in PBSA with 1% NFDm. After three 15-min washes in PBSA with 0.05% Tween-20, the blot was incubated in 0.5 μ g/ml protein A-alkaline phosphatase conjugate (Sigma Chemical Co.) for 1 h. After washing as above, the blot was incubated 5 min in AMPPD chemiluminescent substrate from the Immuno-Lite Substrate Kit (BioRad), wrapped in Saran wrap, and exposed to film.

Northern Analysis. Cytoplasmic RNA was isolated by a mini-prep method (35). Northern blot was performed as described (36). Briefly, 10 μ g RNA was loaded on 0.7% agarose gel and was electrophoresed for 4–7 h. RNA was then transferred to Nytran membranes which were hybridized with probes for VHS107 (37), V κ 22 (38), and for the housekeeping gene CHO-A (39) which was kindly provided by Dr. M. Wilkinson. Autoradiographs were analyzed

by densitometric scanning using a densitometer (model 620; Bio-Rad).

Results

Identification of Secretion-defective Abs. We previously established a panel of T15 Abs restricted to random point mutations in VH CDR2 (S107) to study the effects of V region mutation on Ab function (25). In the course of making transfectomas with the mutated H chain gene constructs, we found that five mutants yielded clones severely impaired in their ability to secrete Ab. As shown in Table 1, mutants M102, M153, M164, M166, and M241 had been transfected several times into an SP2/0 line containing the V κ 22 L chain gene. Of a total of 331 mycophenolic acid resistant clones, only 19 (6%) produced any level of detectable Ab, and, as described below, this level was extremely low. In contrast, transfectants containing WT or other H chains with different CDR2 mutations (M85, M171, and M154) yielded 51 out of 95 (54%) clones that secreted normal levels of Ab.

To determine if other mutations beyond the V region (we had sequenced the V regions of all mutants before transfec-

Table 1. Identification of S107 Low Secretor Mutant Cell Lines

	Expt. 1		Expt. 2		Expt. 3		P3X63Ag8.653	
	mpa ^R	Ig ⁺	mpa ^R	Ig ⁺	mpa ^R	Ig ⁺	mpa ^R	Ig ⁺
Normal secretors								
WT	6*	2 [†]	23	8	ND	ND	33	8
M85	5	3	8	5	ND	ND	ND	ND
M171	5	3	12	6	ND	ND	ND	ND
M154	22	17	14	7	ND	ND	ND	ND
A61T	ND	ND	ND	ND	ND	ND	29	18
Total	38	25	57	26			62	26
Low secretors								
M102	13	–	47	–	ND	ND	ND	ND
M153	22	1, 2 [§]	5	–	32	2, 2 [§]	45	1 [§]
M164	13	2 [§]	ND		40	2 [§]	55	4 [§]
M166	18	2 [§]	33	3 [§]	17	–	24	2 [§]
M241	27	1 [§]	ND	ND	64	2 [§]	19	–
Total	93	8	85	3	153	8	143	7

WT and VHCDR2 mutated T15 transfectants were generated as described in Materials and Methods. Each experiment represents a separate transfection. SP2/0-V κ 22 cells were the recipient cells in experiments 1–3 whereas in the last experiment P3X63Ag8.653 cells were transfected with VHS107 together with V κ 22.

* mpa^R is the number of mycophenolic acid resistant clones (which indicates the presence of transfected H chain DNA) compared to the number of normal antibody secreting clones (Ig⁺) obtained in each transfection.

† Ig⁺ antibody secretion was determined by direct binding ELISA as indicated in Materials and Methods.

§ Transfectants had an OD₄₀₅ reading <0.3 at a time point when WT readings were \geq 1.0. (–) Undetectable.

tion) were affecting Ig expression, up to 500 bp of the 5' flank were sequenced. Only M102 had an altered 5' flank, a deletion of 200 bp that is probably responsible for the non-secretor phenotype, and further studies on this mutant were not performed. In contrast, the only differences distinguishing the other four low secretor mutants from the WT and the normally secreting mutants are the particular CDR2 mutations shown in Fig. 1.

Since the same SP2/0 cell line and the same procedures were used for all transfections, the reproducibility of the low secretor phenotype suggested a defect intrinsic to the mutated H chains. Nevertheless, in order to rule out a possible contribution by the transfection recipient, SP2/0, we made additional transfectants of the four low secretor H chain genes (excluding M102 from further analysis) and the WT S107 H chain gene together with the V κ 22-C κ gene using another myeloma line as recipient, P3X63Ag8.653. As shown in Table 1, the low secretor phenotype was also exhibited in this cell line as only 7 of 143 drug-resistant clones containing the low secretor H chains produced any detectable Ab.

Ig and mRNA Levels of Low Secretor Mutant Cells. Clones that exhibited Ig levels above background during the first screen were saved from each mutant (a few negative clones were also saved). The amount of Ab secreted was quantified. As shown in Table 2, after 4 d of culture, the low secretor clones had very low levels of Ig in the supernatants, ranging from undetectable to 6.8% of the WT transfectant. Further, in other experiments using biosynthetic labeling, Ig H₂L₂ complexes were detected by 6 h in WT supernatants but not in low secretor supernatants (data not shown). Ig was detectable by ELISA in the supernatants of low secretor clones only after several days of culture and the amount was inversely

NORMAL SECRETORS

	50	51	52	a	b	c	53	54	55	56	57	58	59	60	61	62	63	64	65	Ig
WT	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	C	+
M85	-	-	I	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	+
M107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	+
M154	-	-	-	K	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	+
M171	-	-	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
A61T	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	+

LOW SECRETORS

M102*	-	-	-	-	-	-	Y	A	P	Q	-	-	T	-	-	-	-	-	-	-
M153	-	-	-	-	-	-	-	-	-	-	-	-	T	V	-	M	T	-	-	±
M164	-	-	I	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	±
M166	-	-	K	-	-	-	-	-	-	G	-	-	-	Y	M	-	-	-	-	±
M241	-	R	-	-	-	-	E	-	-	S	-	-	-	-	-	-	-	-	-	±

Figure 1. Amino acid sequences of VHS107 CDR2 WT and mutant H chains of normal secretor and low secretor transfectant clones. The remainder of the H and L chain sequences are identical to WT T15. (*Ig*⁺) normal secretion; (*Ig*[±]) secretes <10% of WT; (*Ig*⁻) no Ig detected. Status defined quantitatively in Table 2. M102 was unable to produce any Ig presumably because of a 200-bp deletion in the 5' flanking region as discussed in the text. These sequence data are available from EMBL/GenBank/DDBJ under accession number X79818.

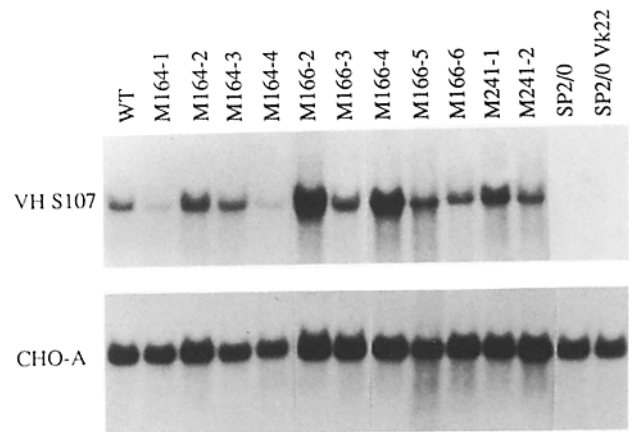


Figure 2. H chain mRNA levels of low secretor clones. Autoradiograms of Northern blots for WT and some of the low secretor clones are shown. RNA samples were first probed for VHS107 (*top*) and the membranes stripped and reprobed for CHO-A (*bottom*) as described in Materials and Methods. Clone names are indicated above each lane.

proportional to cell viability. Thus it is possible that some or all of the mutant clones are true nonsecretors and that Ab is only detectable in the supernatants after cells begin dying. However, since this point has not been formally established, we use the term low secretor to describe the phenotype.

To determine if the defect in Ig secretion is at the transcriptional level, the levels of H and L chain mRNA were examined. Northern analysis with the S107 VH probe revealed that the H chain mRNA levels of most low secretor clones were comparable to or higher than the WT clone (Table 2 and Fig. 2). Moreover, the sizes of the H chain transcripts in the WT and the mutant clones were the same, indicating that gross deletions or truncations were not present. Northern analysis using a V κ 22 probe also indicated that most transfectants had normal levels of L chain mRNA (Table 2). Clearly, transfection had been successful in most instances, suggesting the basis for failure to secrete Ig at normal levels is located at some point after transcription.

Binding Analysis of the Antibody. Anti-idiotypic reagents were used to determine whether gross conformational changes or misfolding might be present in the mutant Abs. The mutant clones were grown for several days to allow Ab to accumulate in the supernatant. Antibodies were purified from the supernatants of clones M153-1, M164-4, M166-1, and M241-2. Three different anti-idiotypic reagents were used to test for conformational changes that might disrupt recognition by the anti-idiotypic. TC54 is a mAb specific for the S107 VH gene product present in T15 even in the absence of L chain (33). The mAb B36-82 and the polyclonal antiserum recognize T15 idiotopes requiring the presence of both T15 VH and V κ 22 (25). All four low secretor mutant Abs were recognized by B36-82 and by the anti-T15 antiserum, indicating that the epitope formed through the interaction of H and L chains was essentially intact (Table 3). M153 and M166 also bound to TC54 (although with reduced avidity) whereas binding to M164 was not detected. It appears that

Table 2. Quantitation of Antibody and Ig mRNA Levels in S107 Low Secretor Mutants

Mutants	Antibody secreted		Ig mRNA	
	ng/ml	Percent WT	VHS107	VK22
WT SP2/0*	736 [‡]	100	1.0 [§]	1.0
WT P3X63Ag	296	100	1.0	1.0
M153-1	2.8	0.4	8.54	11.8
M153-5	–	–	2.88	0
M153-6	2.6	0.4	2.92	6.97
M153-7	2.7	0.4	0.40	ND
M153 P3X63Ag	–	–	0.51	0.16
M164-1	–	–	0.28	0
M164-2	–	–	1.57	0.58
M164-3	2.0	0.3	1.42	0.11
M164-4	12.7	1.7	0.26	2.03
M164 P3X63Ag	7.5	2.8	2.29	3.05
M166-1	15.6	2.1	3.08	2.85
M166-2	28.0	3.8	6.58	2.75
M166-3	8.3	1.1	2.18	3.41
M166-4	50.0	6.8	5.70	2.79
M166-5	4.8	0.7	2.08	3.48
M166-6	–	–	1.25	0.79
M166 P3X63Ag	7.2	2.7	14.02	5.65
M241-1	–	–	3.72	0.72
M241-2	2.6	0.4	1.29	0.81
M241-3	–	–	2.13	1.07
M241-4	–	–	0	0.57
M241 P3X63Ag	–	–	0.36	0.93

* Each clone of the mutants was independently isolated (represents an independent transfection event of the designated mutant H chain).

[‡] The same number of cells (10⁶) was plated for each clone and the supernatants were collected on day 4. Antibody was quantitated on anti-IgG-coated plates using purified isotype-matched antibody as standard. (–) <0.1 ng/ml.

[§] mRNA was determined by Northern analysis using VH S107 or VK22 specific probes. The relative amount of mRNA was estimated by densitometry scanning and normalized to the mRNA signal from the CHO-A housekeeping gene.

^{||} Clones are from P3X63Ag8.653 transfectants.

the epitope recognized by TC54 is vulnerable to alterations caused by CDR2 mutations since the normally secreting mutant, M85, has also lost this epitope. This is consistent with an earlier report that a mutant S107 molecule bearing six VH mutations, one of which was in the CDR2, had also lost its ability to bind to TC54 (40) and with our own findings that other residues in this region may also affect the TC54 epitope (Chen, C., V. A. Roberts, S. Stevens, M. P. Stenzel-Poore and M. B. Rittenberg, manuscript in preparation).

The integrity of the Abs was also tested by their ability to bind to the Ag, PC-histone. Although, as shown previously, mutations in VH CDR2 of T15 frequently result in loss of Ag binding (25), the low secretor M153 Ab isolated

from cell lysates was able to bind PC-protein. These data indicate that the mutated H chains are unlikely to be grossly misfolded, and in the case of M153, can combine with the L chain to form an intact Ab or an L/H dimer. However, the above experiments do not indicate whether the mutated H chains in the low secretors have a normal efficiency or pattern of pairing with the L chain.

Examination of Intracellular Ig Proteins. To determine if the mutant H chain proteins accumulate in the cells and if they assemble with the L chain efficiently, we employed an ELISA to detect intracellular Ig in cell lysates. One representative clone of each low secretor mutant (M153-6, M164-3, M166-3, and M241-2) was analyzed and compared to WT. Cells

Table 3. Binding Features of S107 Low Secretor Mutant Ig as an Indication of Conformational Changes

	Anti-T15 id			
	TC54	B36-82	antiserum	PC-his
WT	+++	+++	+++	+++
M85	-	+++	+++	-
M171	+	+++	+++	-
M154	++	+++	+++	-
M153	++	+++	+++	++
M164	-	+++	+++	-
M166	+	+++	+++	-
M241	ND	+++	+++	-

Binding to various anti-T15 antibodies and to PC-histone was determined by direct binding ELISA as described in Material and Methods. Affinity-purified antibody was used in anti-idiotypic analysis (the low secretor clones M153, M164, M166, and M241 were cultured for an extended period of time in order to accumulate sufficient Ig in the supernatant for testing). Either lysates (M153, M164, M166, and M241) or affinity-purified antibody (M85, M171, and M154) were used in PC-histone binding assays. WT antibody had high OD readings in the PC-histone binding assay regardless of whether it was affinity purified or from lysate. Antibodies were added to the anti-T15 or PC-histone coated plates at a concentration of 100 ng/ml. The OD readings of triplicate wells were averaged and are expressed in a simplified scale: +++ (>0.8); ++ (0.2–0.8); + (0.01–0.2); - (<0.01). The mutant clones M85, M171, and M154 secrete Ig which is not able to bind antigen as described previously (25).

were cultured for 4 h and Ig proteins in the lysates and supernatants were quantified by ELISA. As shown in Table 4, all four mutant clones had a large amount of H chain protein in the cells, ranging from 68 to 185% of the WT level. How-

ever, the intracellular H/L dimers (or tetramers) were greatly reduced in the mutants. Clones M164, M166, and M241 had only 1–8% of the WT level of assembled protein. The exception is M153, which contained intracellular H/L protein at 36% of WT level in keeping with our ability to detect Ag binding activity in this extract.

By 4 h of incubation, the WT clone already had secreted a significant amount of Ab into the supernatant (Table 4). However, at this time point, little or no Ab could be detected in the supernatants of the mutant clones, including M153. Thus, in addition to reduced assembly, a defect in transport/secretion may also account for the low secretor phenotype.

In addition to the ELISA analysis described above, intracellular H and L chains were visualized by Western blot (Fig. 3). The proteins in cell lysates were separated by SDS-PAGE under reducing conditions and H and L chains were detected with anti- γ 2b Abs (which fortuitously cross-react with L chains). H and L proteins were seen in all four low secretor lysates, as well as WT lysates. These proteins run at apparent molecular mass of 54 (H chains) and 26 kD (L chains), and both migrate very similarly to purified MOPC 195 proteins (γ 2b, κ), indicating that no gross alterations of the protein have occurred. Together, these data suggest that the impaired Ab secretion in these mutants is not due to defects in protein synthesis. Rather, deficient H/L assembly or stability may be a major reason for the low secretor phenotype of these mutants. The exception is M153 which appears to assemble but fails to secrete the mutant molecules (consistent with higher levels of intracellular H and L proteins compared to WT in Fig. 3).

Effect of Different L and H Chain D-J Sequences on Secretion. In an attempt to investigate whether the same set of T15 CDR2 mutations would have similar effects on Ab function when associated with a different Ab, the mutated CDR2s were placed in the VH of another mAb, D16, which is en-

Table 4. Quantitation by ELISA of Intracellular Ig Proteins in WT and Mutant Clones

	Lysate				Supernatant	
	H		H+L		H+L	
	ng	%	ng	%	ng	%
WT	16.31	100	9.24	100	31.02	100
M153-6	30.17	185	3.34	36	0.03	0.1
M241-2	16.71	102	0.51	5	-	-
WT	16.67	100	15.70	100	35.35	100
M164-3	12.40	74	1.29	8	-	-
M166-3	11.35	68	0.13	1	-	-

Cells were cultured for 4 h before collection. Cell lysates were prepared as described in Materials and Methods. Intracellular protein was detected by ELISA using either anti- γ 2b (H) or anti- κ (H+L) coated plates, all of which were developed by an anti-IgG2b-alkaline phosphate conjugate. Total ng of Ig protein was determined using isotype-matched, purified antibody as a standard. The number of cells collected for lysis was used to calculate the amount of protein per million cells. Mutants M153, M241 and mutants M164, M166 were tested on separate days, each with a WT control. (-) <0.01 ng per million cells.

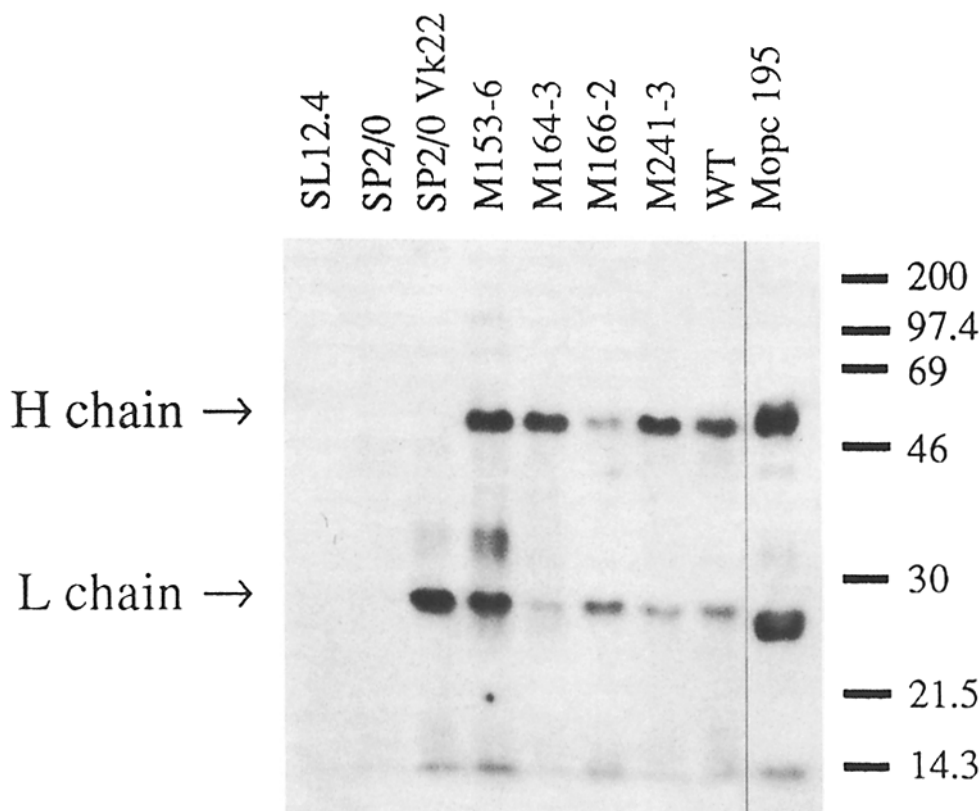


Figure 3. Intracellular H and L chain levels of low secretor clones. SDS-PAGE of lysates and Western blotting with anti-mouse $\gamma 2b$ as described in Materials and Methods. MOPC 195 ($\gamma 2b, \kappa$) is purified antibody from this hybridoma. SP2/0 V $\kappa 22$ is the L chain-containing transfectant used as the recipient of WT and mutant H chains. SL12.4 and SP2/0 (untransfected) are negative control lysates. SL12.4 is an immature murine T cell line provided by Dr. Miles Wilkinson (Oregon Health Sciences University). The additional bands that migrate above the L chain present in SP2/0 V $\kappa 22$ and M153-6 are seen in unreduced Western blots (not shown) and may indicate that reductions of the highly concentrated lysates are not complete. The low mol wt bands at the bottom of the blot represent nonspecific detection. Clone names are indicated above each lane, H and L chain bands are labeled with an arrow, and mol wt markers are indicated on the right.

coded by the same germline S107 VH gene as T15 but differs from T15 in D-J and VL (Fig. 4). Unexpectedly, all four D16 transfectants with the low secretor CDR2 mutations secreted high levels of Ab, ranging from 59 to 141% of the WT level (Table 5).

Thus, the contribution of the CDR2 mutations to low secretion is complex and may be modified through interaction with VL and/or D-J. This result also emphasizes that the CDR2 mutations are unlikely to have caused major structural alterations in VH.

Discussion

In this study we have shown that point mutations in the H chain CDR2 are the cause of defective secretion of an Ig. We drew this conclusion based on several observations. First, L chain-bearing cells transfected with the mutated H chain genes secreted little Ab whereas cells receiving the same H chain gene without mutations or with different mutations in CDR2 secreted normal amounts of Ab. Second, the low

	VH	DH	JH	VL
T15	S107	DYYGSS	YWYFEVWGAGTTVTVSS	V $\kappa 22$
D16	S107	GHYD	YFEYWGQGTTTLTVSS	V $\kappa 1-C$

Figure 4. Comparison of T15 and D16 D-JH sequences; their VH sequences are identical. Amino acid sequences are shown as single-letter code.

secretor phenomenon was observed in repeated transfections with the mutant H chains in two different cell lines and multiple transfectants, excluding the possibility that low secretion was caused by an error in transfection or a defect in the secretory apparatus of the cell itself. Third, no mutations other than those in VH CDR2 were present as determined by se-

Table 5. Effects of L and H Chain D-JH Replacement on Antibody Secretion

Ig combination			Antibody secreted	
VH	D-JH	VL	ng/ml	Percent WT
WT S107	T15	V $\kappa 22$	736.0	100.0
M153	T15	V $\kappa 22$	2.8	0.4
M164	T15	V $\kappa 22$	12.7	1.7
M166	T15	V $\kappa 22$	50.0	6.8
M241	T15	V $\kappa 22$	2.6	0.4
WT S107	D16	V $\kappa 1-C$	642.0	100.0
M153	D16	V $\kappa 1-C$	376.0	58.6
M164	D16	V $\kappa 1-C$	904.0	140.8
M166	D16	V $\kappa 1-C$	483.0	75.2
M241	D16	V $\kappa 1-C$	381.0	59.3

Quantitation of secreted antibody was done as in Table 2.

quencing of the entire V gene. Fourth, transcription or stability of the H chain mRNA appears not to be affected by the mutations since the low secretor mutants express abundant H chain mRNA. Fifth, normal levels of H chain proteins were detected intracellularly; thus the defect appears to be posttranslational.

Somatic hypermutation in Ab V regions represents an important means of generating Ab diversity, in particular, improving Ab affinity after antigenic stimulation (41). Since mutations are introduced randomly into the V region, it is predicted that many mutations would result in nonfunctional Ab such as the low secretors described here. Based on the frequency of invariant residues in the frameworks it has been estimated that 50% of mutations in these regions would result in nonfunctional Ab (42). However, previous efforts to identify such mutations in the VH region have been limited. For example, substitution of the invariant VH residue Cys-92→Tyr or Trp-36→Ala did not show any apparent effect on Ab function (43, 44). Here we show that four mutant Abs with two to four mutations in the VH CDR2 are secretion deficient. These results were unexpected because mutations in the CDRs are usually thought to be permissive due to the highly variable nature of CDR sequences and their importance for generating diversity. These four mutants came from a pool of 46 Abs all of which have point mutations in VH CDR2. Approximately 50% of these mutants had lost or displayed reduced binding to Ag (25). Of the 46 mutant Abs originally described (25), 2 of 9 with four mutations and 1 of 10 with three mutations are shown here to be low secretors. Thus the secretion defect does not reflect the number of mutations but rather their location. The high frequency (4 of 46) of low secretion mutants caused by random CDR2 mutations emphasizes that extensive B cell wastage may occur during the process of somatic mutation and that the creation of nonsecretor variants, in addition to loss of Ag binding, may be a significant factor leading to wastage.

There is increasing evidence that the correct folding and assembly of secreted proteins are necessary for their transport and secretion (45). To determine if the low secretor H chains were misfolded, we tested their binding to several anti-idiotypic Abs. Two of these reagents recognize epitopes provided by the correct combination of H and L chain V regions and the other recognizes structure provided by the H chain V region alone. All four low secretors appear to retain the T15 VH-VL epitope(s) whereas the TC54 VH epitope was lost in one of the three mutants tested. In addition, one of the low secretor Abs, M153, was able to bind Ag. Thus, the low secretor mutations in VH CDR2 do not appear to cause a gross misfolding of the H chain although local conformations such as those recognized by TC54 may be altered.

On the other hand, the levels of intracellular H-L dimer (or tetramer) in these low secretors were greatly reduced (three had <10% of the WT level) even though the amount of unassembled H chains was normal. The L chain expression in these cells should not be an affecting factor since the same SP2/0 cell line (which contains a previously transfected V κ 22

L chain gene) was used in all the transfections in this study and the L chain mRNA expression was normal in most clones of the low secretors. The finding that three mutants exhibit very little intracellular H-L protein could result from inefficient pairing of the mutant H chain with the V κ 22 L chain. Previous *in vitro* experiments have suggested that some H + L pairing may be favored over others (46–48) and, as indicated above, may be affected by somatic mutations (3). Our data may therefore suggest that local structural alterations caused by the CDR2 mutations interfere with H-L assembly and/or the stability of the assembled proteins, and may represent the major cause of the low secretor phenotype in three of the four mutants.

As shown above, mutant M153 makes a reasonable amount of Ab (36% of WT level), but it is not secreted. Thus, a defect in Ab transport/secretion appears likely. The prevailing view of Ab assembly and secretion is that unassembled H chains are retained in the ER via their interaction with the chaperone BiP. BiP is displaced when free L chains become available, and the assembled Ab is transported along the secretory pathway based on signals provided by the L chains (15, 49). Previous studies of λ chain secretion demonstrated that a single substitution at position 15 (Gly→Arg) of λ 2, or position 62 (Phe→Ser) of λ 1 L chains, was sufficient to block secretion (14, 15). Since both of these residues are located on the solvent-exposed surface and are close to each other as predicted by the McPC603 model, it has been proposed that they are included in a surface patch that is required for intracellular Ig transport (15). Here we show for the first time that mutations in the H chain V region may also affect Ab transport. How a local alteration in VH CDR2 could be involved in this process is not clear. The alteration may destroy a transport signal or create a retention signal in VH. Or, it may block or alter the transport signal in the L chain through interactions at the L/H interface. This latter possibility is supported by our finding that the same mutant H chains were secreted when transfected into a cell line (D16) expressing a different V κ gene. The relationship of the two L chains to the CDR2 mutations is not clear at present. V κ 22 alone is not able to be secreted (Scharff, M. D., personal communication as cited in reference 19, and confirmed in our transfectants). In contrast, we have found that the D16 H chain loss variant secretes large amounts of V κ 1c L chain (data not shown). The role played by WT H chain in the secretion of T15 Ab and the role of V κ 1c in rescue of secretion defective mutant H chains remains to be determined. Further investigations on the intracellular transport of the mutant Abs in conjunction with their structural analysis should clarify these possibilities.

It is generally recognized that it is the framework or conserved V region residues that provide the structural basis for proper Ig folding and assembly (50). In the V domain, the CDRs loop out from the β -pleated sheets of the framework regions and form the surface that interacts with Ag. Thus it is surprising to see the low secretor phenotype associated with VH CDR2. Indeed, residues in VL regions known to affect secretion (10, 14–16), and V region positions impor-

tant for VL-VH packing (51) have so far been localized to conserved sites. However, even though there is extensive sequence diversity in the CDR residues, the many known Ab structures actually fit into a small number of main chain canonical structures in the hypervariable regions (50). This CDR structural constraint is believed to be important in forming an Ag binding site. Two of the low secretor mutations described here occur in canonical residues 51 and 52, and several other low secretor mutations are found in a region (56-63) where all known Ab structures exhibit essentially the same main chain conformation (50) (Fig. 1). Thus it is intriguing to consider the possibility that canonical structures in the CDRs may also affect aspects of Igs not associated with Ag binding, such as assembly or intracellular transport. In addition, residues 59 to 65 form the second loop of VH CDR2 which is distant from the binding cavity but in proximity

to the L chain framework 2 region in T15 (25). VL framework 2 is important for formation of the VL-VH interface in McPC603 (51), thus substitutions in VH CDR2 may change the conformation of this loop and interfere with or alter H-L assembly.

Collectively, these low secretors contain amino acid replacements in two general regions of VH CDR2, 51-52 and 58-63, which could potentially perturb several aspects of assembly or transport of the Ig. However other mutants we described previously also had mutations at these same canonical positions (but in different combinations) and yet were secreted normally (25). Further investigation to examine the effects of individual and combined mutations by site-directed mutagenesis and detailed computer modeling will help to clarify these issues and improve our understanding of the structural requirements for Ab assembly and secretion.

We thank Dr. Mary Stenzel-Poore for providing the D16 hybridoma. We also thank her and Arun Bhat for important contributions to the manuscript; and McKay Brown, Dr. Greg Wiens, and Kurt Heldwein for critical review of the manuscript.

This work was supported by grants AI-14985, AI-26827, and EY-07123 from the National Institutes of Health. C. Chen and T. M. Martin were recipients of Tartar Foundation Research Awards and contributed equally to this work.

Address correspondence to Dr. Marvin B. Rittenberg, Department of Microbiology & Immunology, Oregon Health Sciences University, 3181 S. W. Sam Jackson Park Road, Portland, OR 97201-3098. C. Chen is currently at the Department of Molecular Biology, Princeton University, Princeton, NJ 08540. S. Stevens is currently at the Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI 48105.

Received for publication 16 February 1994 and in revised form 6 May 1994.

References

1. Bauml, R., M. Potter, and M.D. Scharff. 1971. Synthesis, assembly and secretion of gamma globulin by mouse myeloma cells. III. Assembly of the three subclasses of IgG. *J. Exp. Med.* 134:1316.
2. Scharff, M.D. 1975. The synthesis, assembly, and secretion of immunoglobulin: a biochemical and genetic approach. *Harvey Lect.* 69:125.
3. Kranz, D.M., and E.W.J. Voss. 1981. Restricted reassociation of heavy and light chains from hapten-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 78:5807.
4. Knittler, M.R., and I.G. Haas. 1992. Interaction of BiP with newly synthesized immunoglobulin light chain molecules: cycles of sequential binding and release. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1573.
5. Alberini, C.M., P. Bet, C. Milstein, and R. Sitia. 1990. Secretion of immunoglobulin M assembly intermediates in the presence of reducing agents. *Nature (Lond.)* 347:485.
6. Sitia, R., M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams, and C. Milstein. 1990. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. *Cell.* 60:781.
7. Hendershot, L., B. Bole, G. Kohler, and J.F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. *J. Cell Biol.* 104:761.
8. Pfeffer, S., and J.E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and golgi. *Annu. Rev. Biochem.* 56:829.
9. Morrison, S.L., R. Bauml, B.K. Birshstein, M.W. Kuehl, J.L. Preud'homme, L. Frank, T. Jasek, and M.D. Scharff. 1974. The identification of mouse myeloma cells which have undergone mutations in immunoglobulin production. *In Cellular Selection and Regulation in the Immune Response.* G.M. Edelman, editor., Raven Press, New York. 233.
10. Mosmann, T.R., and A.R. Williamson. 1980. Structural mutations in a mouse immunoglobulin light chain resulting in failure to be secreted. *Cell.* 20:283.
11. Morrison, S.L., and M.D. Scharff. 1975. Heavy chain-producing variants of a mouse myeloma cell line. *J. Immunol.* 114:655.
12. Sonenshein, G.E., M. Siekevitz, G.R. Siebert, and M.L. Gefter. 1978. Control of immunoglobulin secretion in the murine plasmacytoma line MOPC 315. *J. Exp. Med.* 148:301.
13. Winberry, L., A. Marks, and R. Bauml. 1980. Immunoglobulin production and secretion by variant clones of the MOPC 315 mouse myeloma cell line. *J. Immunol.* 124:1174.
14. Wu, G.E., N. Hozumi, and H. Murialdo. 1983. Secretion of

- a λ_2 immunoglobulin chain is prevented by a single amino acid substitution in its variable region. *Cell*. 33:77.
15. Dul, J.L., and Y. Argon. 1990. A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion. *Proc. Natl. Acad. Sci. USA*. 87:8135.
 16. Dul, J.L., O.R. Burrone, and Y. Argon. 1992. A conditional secretory mutant in an Ig L chain is caused by replacement of tyrosine/phenylalanine 87 with histidine. *J. Immunol*. 149:1927.
 17. Rice, D., and D. Baltimore. 1982. Regulated expression of an immunoglobulin κ gene introduced into a mouse lymphoid cell line. *Proc. Natl. Acad. Sci. USA*. 79:7862.
 18. Pepe, V.H., G.E. Sonenshein, M.I. Yoshimura, and M.J. Shulman. 1986. Gene transfer of immunoglobulin light chain restores heavy chain secretion. *J. Immunol*. 137:2367.
 19. Oi, V.T., S.L. Morrison, L.A. Herzenberg, and P. Berg. 1983. Immunoglobulin gene expression in transformed lymphoid cells. *Proc. Natl. Acad. Sci. USA*. 80:825.
 20. Gardner, A.M., S. Aviel, and Y. Argon. 1993. Rapid degradation of an unassembled immunoglobulin light chain is mediated by a serine protease and occurs in a pre-Golgi compartment. *J. Biol. Chem*. 268:25940.
 21. Morrison, S. 1978. Murine heavy chain disease. *Eur. J. Immunol*. 8:194.
 22. Seligmann, M., E. Mihaesco, J.-L. Preud'Homme, F. Danon, and J.-C. Brouet. 1979. Heavy chain diseases: current findings and concepts. *Immunol. Rev*. 48:145.
 23. Hamers-Casterman, C., T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. Bajjyana Songa, N. Bendahman, and R. Hamers. 1993. Naturally occurring antibodies devoid of light chains. *Nature (Lond.)*. 363:446.
 24. Stenzel-Poore, M.P., and M.B. Rittenberg. 1989. Clonal diversity, somatic mutation, and immune memory to phosphocholine-keyhole limpet hemocyanin. *J. Immunol*. 143:4123.
 25. Chen, C., V.A. Roberts, and M.B. Rittenberg. 1992. Generation and analysis of random point mutations in an antibody CDR2 sequence: many mutated antibodies lose their ability to bind antigen. *J. Exp. Med*. 176:855.
 26. Sharon, J., M.J. Geftter, L.J. Wysocki, and M.N. Margolies. 1989. Recurrent somatic mutations in mouse antibodies to p-azophenylarsonate increase affinity for hapten. *J. Immunol*. 142:596.
 27. Morrison, S.L., M.J. Johnson, L.A. Herzenberg, and V.T. Oi. 1984. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc. Natl. Acad. Sci. USA*. 81:6851.
 28. de St. Groth, S.F., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods*. 35:1.
 29. Stenzel-Poore, M.P., U. Bruderer, and M.B. Rittenberg. 1988. The adaptive potential of the memory response: clonal recruitment and epitope recognition. *Immunol. Rev*. 105:113.
 30. Coffino, P., R. Baumal, R. Laskov, and M.D. Scharff. 1971. Cloning of mouse myeloma cells and detection of rare variants. *J. Cell. Physiol*. 79:429.
 31. Chen, C., M.P. Stenzel-Poore, and M.B. Rittenberg. 1991. Natural auto and polyreactive antibodies differing from antigen-induced antibodies in the H chain CDR3. *J. Immunol*. 147:2359.
 32. Cerny, J., R. Wallach, and G.J. Hammerling. 1982. Analysis of T15 idiotopes by monoclonal antibodies: variability of idiotypic expression on phosphorylcholine-specific lymphocytes from individual inbred mice. *J. Immunol*. 128:1885.
 33. Desaynard, C., A.M. Giusti, and M.D. Scharff. 1984. Rat anti-T15 monoclonal antibodies with specificity for VH- and VH-VL epitopes. *Mol. Immunol*. 21:961.
 34. George, A.J.T., R.C. Foreman, and F.K. Stevenson. 1991. Characterization of a light chain loss variant of the BCL1 lymphoma. *Mol. Immunol*. 28:789.
 35. Wilkinson, M. 1988. RNA isolation: a mini-prep method. *Nucleic Acids Res*. 16:10933.
 36. Buenafe, A., and M.B. Rittenberg. 1989. Molecular analysis and fine specificity of antibodies against an organophosphorus hapten. *J. Immunol*. 143:539.
 37. Early, P., H. Huang, M. Davis, D. Livant, R. Wall, and L. Hood. 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D, and JH. *Cell*. 19:981.
 38. Kwan, S.P., S. Rudikoff, J.G. Seidman, P. Leder, and M.D. Scharff. 1981. Nucleic acid and protein sequences of phosphocholine-binding light chains. *J. Exp. Med*. 153:1366.
 39. Harpold, M.M., R.M. Evans, M.S. Salditt-Georgieff, and J.E. Darnell. 1979. Production of mRNA in chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. *Cell*. 17:1025.
 40. Chien, N.C., R.R. Pollock, C. Desaynard, and M.D. Scharff. 1988. Point mutations cause the somatic diversification of IgM and IgG2a antiphosphorylcholine antibodies. *J. Exp. Med*. 167:954.
 41. French, D.L., R. Laskov, and M.D. Scharff. 1989. The role of somatic mutation in the generation of antibody diversity. *Science (Wash. DC)*. 244:1152.
 42. Shlomchik, M.J., A.H. Aucoin, D.S. Pisetsky, and M.G. Weigert. 1987. Structure and function of anti-DNA autoantibodies derived from a single autoimmune mouse. *Proc. Natl. Acad. Sci. USA*. 84:9150.
 43. Rudikoff, S., and J.G. Pumphrey. 1986. Functional antibody lacking a variable-region disulfide bridge. *Proc. Natl. Acad. Sci. USA*. 83:7875.
 44. Sharon, J. 1988. The invariant tryptophan in an H chain V region is not essential to antibody binding. *J. Immunol*. 140:2666.
 45. Hurlley, S.M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol*. 5:277.
 46. Grey, H.M., and M. Mannik. 1965. Specificity of recombination of H and L chains from human γ G-myeloma proteins. *J. Exp. Med*. 122:619.
 47. Hamel, P.A., M.H. Klein, and K.J. Dorrington. 1986. The role of the VL- and VH-segments in the preferential reassociation of immunoglobulin subunits. *Mol. Immunol*. 23:503.
 48. Roholt, O.A., G. Radzinski, and D. Pressman. 1965. Preferential recombination of antibody chains to form effective binding sites. *J. Exp. Med*. 122:785.
 49. Bole, D.G., L.M. Hendershot, and J. F. Kearney. 1986. Post-translational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol*. 102:1558.
 50. Chothia, C., A.M. Lesk, A. Tramontano, M. Levitt, S. J. Smith-Gill, G. Air, S. Sheriff, E.A. Padlan, D. Davies, W.R. Tulip, et al. 1989. Conformations of immunoglobulin hypervariable regions. *Nature (Lond.)*. 342:877.
 51. Chothia, C., J. Novotny, R. Brucoleri, and M. Karplus. 1985. Domain association in immunoglobulin molecules: the packing of variable domains. *J. Mol. Biol*. 186:651.