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Immunology

Antigen-binding mutants of mouse myeloma cells

(immunoglobulin/binding site/somatic mutation/phosphocholine/generation of antibody diversity)

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ABSTRACT **A cultured mouse myeloma cell line, S107, that secretes an IgA phosphocholine-binding immunoglobulin has been cloned in soft agar and overlaid with phosphocholine-hemocyanin. Spontaneous mutants that secrete immunoglobulin with a decreased ability to precipitate antigen were detected with this plate assay and occur at a very high frequency. From one such mutant, phenotypic revertants arise spontaneously with a frequency of 0.28–2.8%. This mutant and one of its revertants were studied, and they were found to differ from the parent and from each other serologically and in antigen binding. While it is not yet clear whether these findings bear any relationship to the normal generation of antibody diversity, they do indicate that it is possible to generate antigen binding diversity in somatic cells.**

In recent years, biologists have devoted considerable thought and effort to trying to understand how individuals produce the large number of antibodies that they require to respond to a seemingly limitless number of different antigens (1). Chemical studies of naturally occurring antibodies and myeloma proteins have shown that the recognition of many different antigens depends on the generation of an enormous amino acid sequence diversity in small areas of the NH_2-terminal part of immunoglobulin polypeptide chains (2–4). In the heavy (H) chain, these areas, called the hypervariable regions, are found between residues 31 and 35, 50 and 65, and 95 and 107 (4). The evidence that these hypervariable regions form the antigen-binding site comes from correlations between sequence and specificity (4), affinity labeling with antigens (5), and studies on the three-dimensional structure of antibodies (6, 7). There has been a great deal of discussion about whether the generation of this sequence diversity occurred during evolution, with genes for each of these many sequences being inherited through the germ line, or if diversity is generated somatically through the mutation of a limited number of germ line genes (1).

A few investigators have tried to demonstrate a somatic generation of antibody diversity by looking for structural gene mutations in cultured immunoglobulin-producing cells. In fact, in cultured mouse myeloma cells, the production of immunoglobulins is very unstable (8, 9). Spontaneous variants arise at a rate of 10^{-3}/cell per generation, and certain mutagens increase the frequency of variants to as high as 2–6% of surviving clones (10, 11). While many of these spontaneous and mutagen-induced variants have lost the ability to produce immunoglobulin, approximately 40% of the mutagen-induced variants do produce heavy chains that differ structurally from the parental heavy chains (12, 13). Milstein and his colleagues have used amino acid and nucleic acid sequence studies to show that frameshift, missense, and nonsense mutations occur spontaneously in their cultured cell lines (8). The genetic instability of immunoglobulin production is not due to a general instability in mouse myeloma cells because mutants resistant to 6-thioguanine and bromodeoxyuridine arise in the same cells at a rate of 10^{-6}–10^{-7}/cell per generation (10).

Although this enormous genetic instability in cultured myeloma cells might have suggested a somatic generation of antibody diversity, all of the mutations reported so far (8, 12, 13) have been in the COOH-terminal or constant region of the molecule, which is highly conserved in normal antibodies and myeloma proteins (2). This was especially perplexing because Cotton *et al.* (14) screened 7000 clones by isoelectric focusing and Kohler and Milstein screened hundreds of clones for loss of ability to lyse sheep erythrocytes (15) without detecting a single mutation in the NH_2-terminal region of the molecule. We have recently reexamined this problem using the S107 mouse myeloma cell line, which produces an IgA κ immunoglobulin that binds the hapten phosphocholine (PC) (16). In this communication we report that mutants that have a change in their ability to bind PC conjugated with keyhole limpet hemocyanin (KLH) occur at a high frequency. We have also found that one such antigen-binding mutant reverts at a high frequency. This has led us to conclude that, at least in this model system, it is possible to generate diversity of antigen binding in somatic cells.

MATERIALS AND METHODS

Cloning and Detection of Variants with Antibody and Antigen. The S107 cell line was kindly provided by Melvin Cohn of the Salk Institute. The cells were grown in suspension culture in Dulbecco's modified Eagle's medium (Gibco) supplemented with 20% horse serum. Cells were cloned in 60-mm tissue culture dishes (Falcon) in soft agar and variants were identified as described previously (17) except that Sea Plaque agarose (Marine Colloids, Inc.) and rat embryo feeders were used. To identify variants that had lost the ability to secrete IgA, clones were overlaid with agarose containing antibody raised against the protein from the IgA-producing Adj PC6A myeloma, which does not bind PC. This antiserum did not react with κ light (L) chains. Variants were identified by screening the dishes under medium power with an inverted microscope to look for clones that were not surrounded by an antigen-antibody precipitate (17).

To detect S107 clones secreting immunoglobulin that did not precipitate with PC-KLH, dishes containing small clones were overlaid with 1 ml of agarose containing the hapten-protein conjugate PC-KLH at 1 mg/ml. In order to couple the PC to KLH, 17 μmol of diazophenylphosphocholine prepared from *p*-nitrophenylphosphocholine (Biosearch, San Rafael, CA) as described by Chesebro and Metzger (18) was incubated with 144 mg of KLH (Calbiochem) in 0.08 M $Na_2B_4O_7$/0.35 M $KHCO_3$ at 4°. The pH was monitored and adjusted to 9.0 with

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Abbreviations: PC, phosphocholine; KLH, keyhole limpet hemocyanin; H and L, immunoglobulin heavy and light chains.

1 M NaOH for 30 min and then the incubation was continued with stirring overnight. Excess diazophenylphosphocholine was removed by dialysis against 0.025 M Tris·HCl, pH 8, in 0.15 M NaCl. Precipitated KLH was removed by centrifugation. The soluble PC-KLH was estimated to contain 120 nmol of PC per mg of KLH.

While variants that did not give visible precipitates with PC-KLH could be detected by overlaying with PC-KLH alone, the period of optimum staining was short and the amount of precipitate small. We found that we could increase the ease of scoring clones by adding a subliminal amount of antibody against the COOH-terminal part of the IgA heavy chain to the agarose at the time of cloning. This subliminal amount of antibody was chosen by testing different dilutions of a given antiserum and using a dilution that resulted in a just-detectable amount of antigen–antibody precipitate around clones containing 2 to 4 cells but no visible precipitate around larger clones. As more immunoglobulin was secreted by the clone and the IgA–anti-IgA precipitate was dissolving in antigen excess, the clones were overlaid with PC-KLH. We assume that this sequence of events caused IgA to accumulate around the clones rather than diffuse away. When the PC-KLH is added, larger amounts of secreted anti-PC are available to form a more visible precipitate. Controls were always carried out with the subliminal dose of antibody and no PC-KLH to be sure that we were really looking at PC-KLH reactivity.

Between 250 and 1000 clones were scored per plate. Subclones and presumptive variants were removed from the agarose and grown up to mass culture as described previously (17).

Labeling of Cells and Electrophoretic and Chromatographic Analysis of Labeled Protein. Logarithmically growing cells were incubated with ^{14}C- or ^{3}H-labeled valine, threonine, and leucine, and the intracellular or secreted immunoglobulins were specifically precipitated and analyzed on acrylamide gels containing sodium dodecyl sulfate. For the analysis of the tryptic–chymotryptic peptides, secreted immunoglobulins were completely reduced and alkylated, heavy and light chains were purified on cylindrical sodium dodecyl sulfate gels, and the digests were prepared and analyzed by ion exchange chromatography using pyridine acetate buffer. The details of these techniques have been described previously (19, 20).

Purification of Wild-Type and Mutant Proteins. Wild-type, mutant, or revertant cells were injected intraperitoneally into pristane-primed BALB/c mice (21). The ascites fluid was collected and the immunoglobulin was purified by affinity chromatography on a PC-Tyr-Gly-Sepharose 4B column followed by elution with PC and dialysis (22). The purity of each protein preparation was confirmed using both sodium dodecyl sulfate/acrylamide gels to examine the size of the protein and nondissociating Tris/glycine gels (23) to analyze the charge homogeneity of the preparation. No contaminating proteins were detected.

Radioimmunoassay with Anti-variable Region Antisera and with PC-KLH. Rabbits were immunized with purified S107 and early high titer bleedings were used to prepare two types of antibody specific for the S107 variable region. Antiserum reactive with the PC-binding site was prepared as described by Claflin and Davie (24). Briefly, the S107 protein was coupled to Sepharose 4B and the rabbit antibody raised against the S107 protein was passed through the column. After thorough washing, those antibodies that were bound to the PC-binding site were eluted with a large excess of PC. The hapten was then removed by extensive dialysis (24). Antiserum specific for the S107 variable region was prepared by successive absorptions with Sepharose 4B coupled with: (*i*) W3129 protein, an α1,6-dextran-binding IgA κ (kindly provided by Sherie Morrison); (*ii*) normal mouse serum; and (*iii*) a mixture of protein from the MOPC 511 and McPC 603 tumors, both of which produce IgA κs that bind PC but differ from S107 in their idiotype and light chains (25). The absorbed antiserum reacts in double diffusion in agar (Ouchterlony) with S107 protein and with protein from the HOPC-8 tumor, an IgA κ myeloma that binds PC and has the same variable region serology as S107, but does not react with other IgA or IgG myelomas.

Radioactive S107 protein was endogenously labeled by incubating cells with [^{35}S]methionine and purifying the secreted protein with PC-Sepharose (22). Radioimmunoassays were performed using an adaptation of a solid-phase assay employing polyvinyl microtiter plates (26). Amounts of antiserum or PC-KLH sufficient to bind 4000 cpm of the ^{35}S-labeled S107 protein were used to coat each well. The wells were washed with phosphate-buffered saline, incubated for 30 min at 25° with 1% bovine serum albumin in phosphate-buffered saline, and washed again. Samples containing 4500 cpm of ^{35}S-labeled S-107 protein were mixed with dilutions of unlabeled wild type, mutant, revertant, or control proteins, all in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% normal rabbit serum. One hundred microliters of duplicate mixtures was added to microtiter wells and incubated overnight at 4°. For PC-KLH binding, labeled S107 and unlabeled proteins were mildly reduced to H_2L_2 monomers before they were added to the microtiter wells. After incubation, the wells were washed several times, first with phosphate-buffered saline and then with distilled water. The wells were cut out of the plates, and their radioactivities were measured in a liquid scintillation counter. After the background had been subtracted, the results were expressed as percent of the maximum ^{35}S-labeled S107 protein bound. Duplicates did not differ by more than 5%.

RESULTS

When a recently isolated subclone of S107 was cloned in soft agar and overlaid with PC-KLH, most of the clones were surrounded by a visible antigen-antibody precipitate. However, a number of clones were unstained (experiment 1, Table 1). Subclones that did stain with antigen were recovered and recloned. Unstained clones were again present (experiments 2, 3, 4 and 5, Table 1). Because a lack of visible antigen–antibody precipitate could result from blocks in immunoglobulin synthesis, assembly, or secretion, as well as from a change in the antigen-binding capacity of the S107 protein, one set of plates containing S107 clones was overlaid with antibody against IgA while another set of plates containing cells from the same population was overlaid with PC-KLH (experiment 5, Table 1). As would be expected from our previous studies with other cell lines (9), S107 spontaneously generated variants that no longer secreted IgA molecules. However, the frequency of

Table 1. Frequency of variants

Exp.	Subclone*	Overlay	Unstained/stained	%
1	S107.3.4	PC-KLH	4/1652	0.24
2	S_1 (4 weeks)	PC-KLH	16/3740	0.43
3	S_1S_2 (5 weeks)	PC-KLH	9/2348	0.38
4	S_1S_4 (16 weeks)	PC-KLH	20/1107	1.81
5	S_3 (8 weeks)	PC-KLH	10/1105	0.91
		Anti-IgA	4/1339	0.29

* Each subclone was freshly isolated; parentheses indicate time after isolation that the experiment was performed.

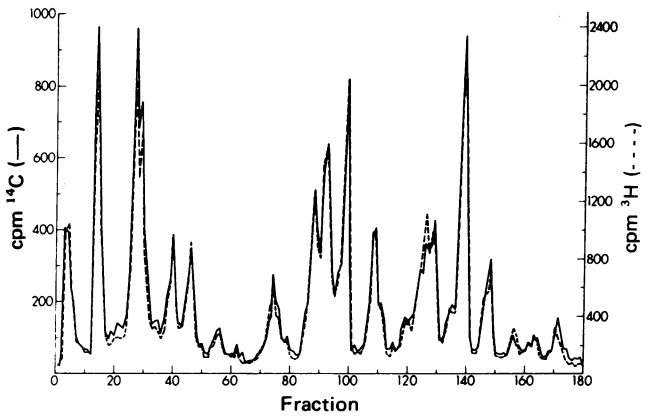

FIG. 1. Wild-type (^{14}C, —) and mutant U_1 (^{3}H, - - -) cells were labeled with radioactive valine, threonine, and leucine. Immune-precipitated secreted light chains were digested with trypsin and chymotrypsin and peptides were analyzed by ion exchange chromatography.

variants that did not form precipitates with PC-KLH was three times higher than the frequency of IgA-negative variants (experiment 5, Table 1), suggesting the existence of a large number of variants that continued to secrete IgA but had a change in antigen binding.

A number of clones that did not react with PC-KLH in the plate assay were recovered, grown to mass culture, and characterized in more detail. For example, in one experiment, ten unstained clones were recovered. Three no longer synthesized IgA heavy chains. The remaining seven all synthesized and secreted IgA. Both the intracellular and secreted IgA of the parental clone precipitated with PC-KLH in agar diffusion (Ouchterlony). The intracellular and secreted material of the seven unstained clones did not react with PC-KLH under identical conditions. The secreted material from all seven unstained clones was shown to retain some PC-binding activity by the more sensitive hemagglutination assay. This preliminary analysis confirmed that most of the variant clones did secrete IgA but that the IgA differed from the parent in its ability to bind PC. This was not due to differences in the amount of immunoglobulin secreted because medium from mutants and the parent contained similar amounts of IgA as assayed by radial immunodiffusion. IgA-positive PC-KLH-negative variants from three independent experiments were recloned and then the cells were incubated with radioactive amino acids and their intracellular and secreted immunoglobulins were analyzed on polyacrylamide gels containing sodium dodecyl sulfate. All but one synthesized heavy and light chains that comigrated with those of the parent and assembled their chains into higher polymers similar to those produced by the parent (data not shown).

One of the variants, S1U9, synthesized a heavy chain that spurred with the parent when reacted with anti-IgA on Ouchterlony analysis, indicating that it had lost some antigenic determinants. Gel electrophoresis of the IgA secreted by this variant revealed that the heavy chain was smaller than that of the parent and was disulfide linked to the light chain, forming an HL half molecule. Similar defective IgAs are produced by mouse myelomas that synthesize IgA heavy chains with a COOH-terminal deletion; such IgAs lack inter-heavy chain disulfide bonds and form abnormal disulfide bonds between their heavy and light chains to produce HL half molecules (21, 29, 30). We assume that this mutant has a related defect, although it has not yet been studied further. The apparent change in antigen binding in S1U9 could be due to the fact that it is

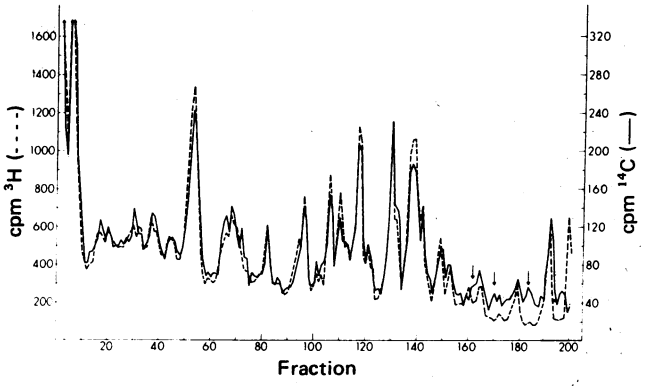

FIG. 2. Heavy chains of wild-type (^{14}C, —) and mutant U_1 (^{3}H, - - -) were analyzed as the light chains were (Fig. 1). Arrows indicate differences.

monovalent for PC and therefore could not form the lattice necessary to produce a visible antigen–antibody precipitate.

One mutant (U1) was studied in more detail. Its endogenously labeled heavy and light chains were purified and digested with trypsin and chymotrypsin, and the resulting peptides were compared with those of the parent. The mutant and parental light chains appear identical (Fig. 1). The heavy chains are also largely similar but there are a few differences (Fig. 2), which were seen in two independent analyses.

The mutant cells were recloned and injected into the peritoneal cavity of pristane-primed mice. The mutant protein was purified from the resultant ascites fluid and its ability to bind to PC was compared with that of the parent. With a radioimmunossay, it can be seen that the mutant binds to PC but that approximately a 10-fold excess of mutant protein is required to compete with the parental protein for 50% of the hapten sites (Fig. 3).

Antibody specific for the S107 variable region was prepared as described in *Materials and Methods*. When this antibody was used in a solid-phase radioimmunoassy, the mutant U1 was indistinguishable from the parent (Fig. 4*A*). However, when PC-binding-site-specific antibody was used, the mutant was significantly less reactive than the parent (Fig. 4*B*). MOPC 511 protein, which binds PC, but has a different variable region serology from S107, was included as a control for these two assays.

These chemical, antigen-binding, and serological studies all suggest that the mutant has undergone a small change in its

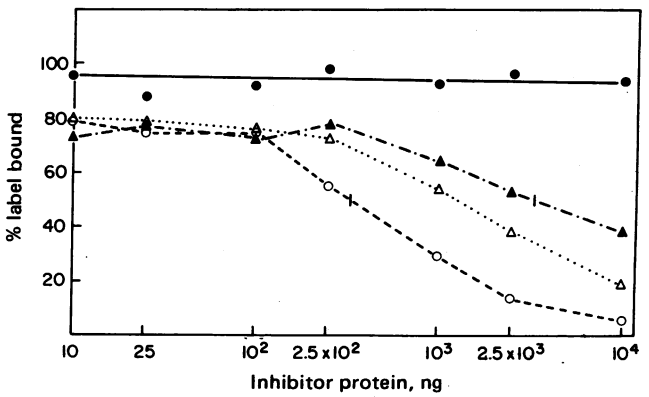

FIG. 3. The competitive inhibition of binding of endogenously labeled [^{35}S]methionine wild-type protein to PC-KLH absorbed onto microtiter plate wells. The figure shows the effect on label bound of increasing amounts of unlabeled wild-type (○), mutant (▲), revertant (△), or W3129 (●) protein.

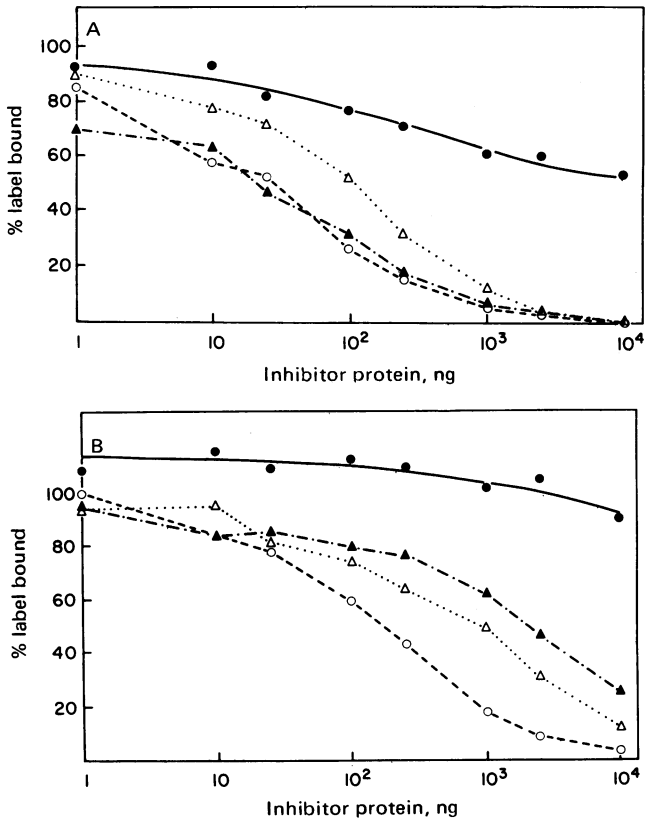

FIG. 4. Competitive inhibitions of binding of endogenously labeled [^{35}S]methionine wild-type protein to (*A*) absorbed rabbit anti-variable region serum or (*B*) PC-binding-site-specific antibody adsorbed onto microtiter plate wells. See *Materials and Methods* for preparation of antisera and assay procedure. The figure shows the effect on label bound of increasing amounts of unlabeled wild-type (○), mutant (▲), revertant (△), or MOPC 511 (●) protein.

amino acid sequence, which has affected the PC-binding site. In order to see if reversion could be obtained, a fresh subclone of mutant U1 was recloned and overlaid with PC-KLH. As expected, most of the clones were unstained, but revertant clones that stained were present at a high frequency (Table 2). Presumptive revertants were recovered, recloned, and grown to mass culture. Their phenotype has been confirmed by Ouchterlony analysis. Purified protein has been prepared from one revertant. The revertant makes normal-sized heavy and light chains. Its PC-KLH binding capacity is intermediate between that of the wild type and the mutant from which it was derived (Fig. 3). When examined with either variable-region specific (Fig. 4*A*) or PC-binding-site-specific (Fig. 4*B*) antiserum, the revertant is seen to differ from both the mutant and the parent. These results suggest that revertants occur at a high frequency and that at least some of these are phenotypic revertants that have undergone compensatory changes resulting in an alteration in antigen binding and increased antigen precipitation.

DISCUSSION

The results presented above show that S107.3.4 spontaneously generates mutants at a very high frequency. A number of pieces of evidence suggest that there has been a change in the PC-binding site of the mutant studied in detail. The mutant immunoglobulin does not precipitate with PC-KLH either in the overlay assay or on Ouchterlony analysis; it hemagglutinates PC conjugated to sheep erythrocytes but at a much lower titer than the parent, a 10-fold excess of mutant protein is required to compete with the parent for PC-KLH binding sites in a radioimmunoassay, and the mutant differs from the parent when analyzed with binding-site-specific antibody. Because a great deal is known about the sequence (32), contact residues (33), and three-dimensional structure (34) of the PC-binding site, amino acid sequence studies should reveal whether there are structural changes in the binding site or elsewhere in the molecule.

Table 2. Frequency of revertants

Exp.	Subclone	Overlay	Stained/ unstained	%
1	U_1U_1 (4 weeks)	PC-KLH	53/2332	2.28
2	$U_1U_1U_4$ (4 weeks)	PC-KLH	3/1075	0.28
3	$U_1U_1U_3$ (16 weeks)	PC-KLH	28/1014	2.76

An antigen-unstained clone U_1 was recovered. On recloning, an unstained subclone U_1U_1 was recovered. In independent experiments recloning of this and two of its subclones gave antigen-stained clones in the frequencies shown.

Because reversion here is measured by a positive event, its frequency will be a valid measure of the rate of mutation in S107. This aspect of the assay may also explain this high frequency when compared with that of forward mutation. These results indicate that, at least in S107.3.4, spontaneous mutations occur at avery high rate. It is not clear whether this genetic instability is related to any of the normal events in the genetic control of antibody production. In fact, it would be surprising to find the normal generation of antibody diversity occurring in differentiated plasma cells. However, we are certainly observing a "somatic generation of antigen binding." One of the major characteristics usually predicted for the normal generation of antibody diversity is that the mechanism or mechanisms responsible will act selectively on the hypervariable regions (1). In these studies we have observed variants that have lost the ability to produce heavy chains. In other studies, constant region mutants have been shown to occur at a high frequency (8, 9), and one possible constant region mutant, S1U9, was recovered in these studies. Because the mutants and revertants described in this study were identified by changes in antigen binding, we cannot rule out the possibility that mutations in parts of the molecule not involved in antigen binding are occurring at an equally high rate.

Finally, it is not obvious why previous studies of mouse myeloma cells have not revealed variable region mutants. Isoelectric focusing may not be sensitive enough, especially if changes are largely conservative. Hemolysis (15) may be too sensitive in that a 10-fold change in binding may not result in loss of ability to bind and lyse erythrocytes. The genetic instability we have observed is not unique to S107, because we have found a high frequency of MPC-11 mutants that secrete IgG_{2b} immunoglobulin that has decreased reactivity with antivariable region antibody (B. Dharmgrongartama and M. D. Scharff, unpublished data).

In conclusion, the S107.3.4 cell line frequently generates mutants that have changes in antigen binding. This suggests that it will be possible to generate antibody diversity in somatic cells in culture. Detailed analysis of these mutants and revertants should allow us to learn more about the relationship between idiotype, antigen binding, and sequence and to determine whether the mechanism responsible for this somatic instability is related to the normal generation of antibody diversity.

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