

Identification of mutant monoclonal antibodies with increased antigen binding

(hybridomas/somatic mutation/avidity)

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ABSTRACT Sib selection and an ELISA have been used to isolate hybridoma subclones producing mutant antibodies that bind antigen better than the parental monoclonal antibody. Such mutants arise spontaneously in culture at frequencies of $2.5\text{--}5 \times 10^{-5}$. The sequences of the heavy and light chain variable regions of the mutant antibodies are identical to that of the parent and the K_a values of the mutants and the parent are the same. The increase in binding is associated with abnormalities of the constant region polypeptide and probably reflect changes in avidity of these antibodies.

In the course of the immune response to foreign antigen, antibodies with progressively higher affinities are found in the circulation (1). Recent studies of families of monoclonal antibodies encoded by the same germ-line variable (V) region genes (2–9) have shown that this affinity maturation is largely due to somatic hypermutation (2, 10) of the heavy and light chain V regions and subsequent selection, presumably by antigen and accessory cells, for the B cells that are producing the highest-affinity antibodies (11–16). The rate of V region mutation *in vivo* has been estimated to be as high as 10^{-3} per base pair per generation (6, 7). Further, the available data suggest that somatic mutation is limited to the V region and its immediate flanking sequences and does not extend into the constant (C) region of the immunoglobulin gene (10, 16–18).

Because it is difficult to study the molecular mechanisms of somatic hypermutation *in vivo*, investigators have attempted to identify cell lines undergoing a high rate of V region mutation in tissue culture (19). The S107 mouse myeloma cell line frequently and spontaneously generates V region mutations that result in a loss of antigen binding (20–23). However, heavy chain C region mutations also occur frequently in the S107 cell line (24). The 18-81 pre-B-cell line undergoes a high rate (10^{-5} per base pair per generation) of heavy chain V region mutation in culture (25), whereas C region mutations appear to occur much less frequently (26).

Hybridoma cell lines producing monoclonal antibodies have also been examined for V region mutations (19). Bruggermann *et al.* (27) have shown that hybridoma cells can undergo somatic V region diversification *in vitro* but these occurred at very low frequencies of $\approx 10^{-7}$ per base pair per generation. Others who have looked for mutations in the immunoglobulins of either hybridoma or myeloma cells have been unable to find V region mutations but have found that heavy chain C region mutations occur frequently (19). In many studies this might be explained by the detection systems used. However, Köhler and Shulman (28) developed a technique that selected for cells producing antibodies that had lost the ability to bind antigen. Even with this

technique all of the mutants identified had mutations in the heavy chain C rather than the V region (29, 30).

It seemed possible that the low frequency of V region mutations in hybridoma cell lines was due to the negative detection systems that had been used rather than to a seemingly paradoxical stability of the V region genes. Since it would also be very useful if monoclonal antibodies with increased binding could be obtained from hybridomas making low-affinity antibodies, we have developed a “positive” detection system that will identify even rare subclones that are making monoclonal antibodies with increased binding. This report describes the detection system and the identification of such higher-binding mutants. Further, we show that the mutants with greatly increased antigen binding that we have obtained have wild-type heavy and light chain V regions and altered C regions.

MATERIALS AND METHODS

Cell Lines. The anti-*p*-azophenylarsonate (Ars), IgG1 κ hybridomas 36-65 ($K_a = 2.5 \times 10^5 \text{ M}^{-1}$), 45-223 ($K_a = 5.0 \times 10^6 \text{ M}^{-1}$), and 36-71 ($K_a = 4.5 \times 10^7 \text{ M}^{-1}$) (12) were obtained by fusing Ars-immunized splenic lymphocytes from A/J mice to the SP2/0 myeloma (31). The cell lines were maintained in Dulbecco's modified Eagle's medium (GIBCO) containing 15% fetal calf serum, 5% NCTC-109 (M. A. Bioproducts, Bethesda, MD), 1% nonessential amino acids, and penicillin/streptomycin. All cell cultures were incubated at 37°C in a humidified atmosphere of 8–10% CO₂ in air.

Sib Selection Technique. Some aspects of this approach have been described for identifying class-switch variants (32). Briefly, cells were plated into each well of a 96-well microtiter plate (Falcon) at 250–500 cells per 100 μl of medium. A total of 20–30 plates was set up for each experiment so that a total of $0.5\text{--}1.5 \times 10^6$ cells could be screened for mutants in a single experiment. After the cells had grown to half-confluence, they were fed (100 μl per well) and assayed 2 days later. Culture supernatants were removed from each well by using a 96-well dispenser (Handi-Spense, Sandy Spring, Vineland, NJ) and assayed by an ELISA described below. Cells from wells that were positive by ELISA were respread in another sib selection at 25–50 cells per well and then cells from positive wells were spread at 5–10 cells per well. After this point the cells were cloned by limiting dilution and the mutant cells were isolated by repeated cloning in soft agar (33).

ELISA for Mutants with Increased Antigen Binding. Different concentrations of Ars were conjugated to bovine

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Abbreviations: V, variable; C, constant; Ars, *p*-azophenylarsonate.
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serum albumin (hereafter referred to as albumin) according to a standard protocol (34). The ratio of Ars to albumin in each preparation was estimated by using the method of Rao *et al.* (35). Five preparations were made with ratios of 2.40, 1.80, 0.70, 0.40, and 0.15 mol of Ars per mol of albumin. Each preparation was tested as antigen in an ELISA to identify the preparation of Ars-albumin that resulted in the greatest differential binding between the low-, intermediate-, and high-affinity antibodies, 36-65, 45-223, and 36-71. The 0.4 mol of Ars per mol of albumin preparation at 10 $\mu\text{g}/\text{ml}$ gave the best results (see Fig. 1) and was used as antigen in the assay for the selection of higher-binding mutants.

Each well of an ELISA plate (Costar, Cambridge, MA) was coated with Ars-albumin [10 $\mu\text{g}/\text{ml}$ in phosphate-buffered saline (PBS)] for 2 hr at room temperature and blocked with 1% albumin in PBS. Culture supernatants from the sib selection were added (50 μl per well) to the ELISA plate. After incubation for 1.5 hr at 37°C in an 8% CO₂ in air incubator (to keep a neutral pH), an alkaline phosphatase-labeled rabbit anti-mouse IgG (Zymed Laboratories, Burlingame, CA) diluted in 1% albumin/PBS was added (50 μl per well). Plates were incubated for 1.5 hr at 37°C. Substrate (*p*-nitrophenyl phosphate, disodium) was dissolved in a bicarbonate buffer (0.001 M MgCl₂/0.05 M Na₂CO₃, pH 9.8) and added to each well (50 μl per well). Plates were read on an ELISA reader (Flow Laboratories) and wells giving signals 50% or more above background were scored as positive.

Culture supernatants from hybridomas producing mutant antibodies were collected and dialyzed against PBS containing sodium azide (10 mM). The immunoglobulin was quantitated (see below) and assayed in the same ELISA as described above.

Immunoglobulin Quantitation. An inhibition ELISA was used with a rat anti-mouse κ light chain monoclonal antibody (36). The rat monoclonal antibody (187.1) was initially titered in wells coated with 1 μg of IgG1 protein per ml (MOPC 21, Bionetics, Kensington, MD) to find the dilution giving 90% binding. Hemagglutination plates, precoated with 1% albumin/PBS and washed with PBS, were used to incubate the rat monoclonal antibody with the various antibody preparations. MOPC 21 immunoglobulin was used for the standard curve. Briefly, the antibody preparations and the MOPC 21 standard (5 $\mu\text{g}/\text{ml}$) were serially diluted (50 μl per well in 1% albumin/PBS) in 96-well hemagglutination plates. The rat monoclonal antibody was added (50 μl per well in 1% albumin/PBS) and the plates were incubated at 4°C overnight. The antibody/monoclonal antibody mixtures were transferred (50 μl per well) and incubated for 1 hr at 37°C in an ELISA plate that had been coated with MOPC 21 (100 μl of 1 $\mu\text{g}/\text{ml}$ in PBS) and blocked with 1% albumin/PBS. The plates were developed with an alkaline phosphatase-labeled anti-rat IgG (50 μl per well in 1% albumin/PBS) (KPL Laboratories, Gaithersburg, MD) and substrate (50 μl per well). The quantity of antibody was determined from the linear part of the standard curve.

NaDodSO₄/PAGE of Biosynthetically Labeled Immunoglobulin. The conditions for biosynthetic labeling of immunoglobulins and running of Tris/glycine/polyacrylamide gels have been described in detail elsewhere (37, 38). Proteins were labeled with [³⁵S]methionine, immunoprecipitated on Ars-Sepharose, and eluted with 1% NaDodSO₄ (100°C, 3 min).

Sequencing of mRNA. The nucleic acid sequences of the antibody V regions were determined either by base-specific cleavage of cDNAs whose synthesis was primed by end-labeled oligonucleotides (39) or by oligonucleotide-primed dideoxy sequencing as modified by Geliebter *et al.* (40). Since the sequences presented here were done by the latter method, only it will be described here. RNA was isolated by

the guanidinium isothiocyanate method (41) and poly(A)⁺ RNA was purified over an oligo(dT) column. For each sequencing reaction, $\approx 15\text{--}20$ μg of poly(A)⁺ RNA was used. Oligonucleotides were synthesized in our Oligonucleotide Shared Facility and two oligonucleotides were used for the sequencing of each V region. For the heavy chain, an IgG1-specific oligonucleotide with the sequence 5' AGATGGGGGTGTCGTTTGGC 3' was synthesized to hybridize to the 5' end of the CH1 domain, and a V_HId^{CR} oligonucleotide with the sequence 5' GTACTTAGTATAACCATT 3' was synthesized to hybridize to the second hypervariable region of the V region. To sequence the κ light chain, an oligonucleotide with the sequence 5' TGGATGGTGGGAA-GATG 3', which was complementary to the C region sequence 23 bases downstream from the joining region, and another oligonucleotide with the sequence 5' TGGGAC-TCTGAGTGTA 3', which recognizes the middle of the V_κ10-Ars-A gene, were synthesized.

Determination of Association Constants. The affinities of 36-65 and the mutant proteins were determined by the fluorescence quenching technique as described by Rothstein and Gefter (12). The measurements were performed on purified proteins at 22°C with Ars-coupled *N*-acetyl-*L*-tyrosine in the laboratory of Tim Manser (Princeton University).

RESULTS

Sib Selection for Higher-Binding Mutants. To isolate mutant hybridomas producing antibodies with increased antigen binding, it was necessary to develop an appropriate detection system. Studies with two different haptenic systems have shown that low-affinity antibodies do not bind appreciably when hapten is present at a low density on carrier protein, whereas antibodies with a 10- to 20-fold higher affinity do bind effectively to such hapten protein conjugates (12, 42). Presumably, the low density of hapten causes IgG antibodies to bind by only one arm and low-affinity antibodies do not bind well enough to be retained on the plate, whereas higher-affinity antibodies remain bound to antigen and can be detected with a second antibody. This suggested that an ELISA could be constructed by using a hapten density that would allow us to detect small amounts of higher-affinity antibody in the presence of a large excess of lower-affinity wild-type antibody.

To explore this possibility, we examined the binding of a family of IgG1 monoclonal anti-Ars antibodies to an Ars-albumin conjugate of 0.4 mol of Ars per mol of albumin that was absorbed to ELISA plates at a concentration of 10 $\mu\text{g}/\text{ml}$ (Fig. 1). The 36-65 monoclonal antibody expresses the germ-line sequence of the CRI_a idotype of A/J mice and has a reported K_a of $2.5 \times 10^5 \text{ M}^{-1}$ (12). Monoclonal antibodies 45-223 and 36-71 have the same idotype and are derived from the same germ-line genes but have accumulated a number of somatic mutations *in vivo* and have K_a values of $5.0 \times 10^6 \text{ M}^{-1}$ and $4.5 \times 10^7 \text{ M}^{-1}$, respectively (12). These mutants show that somatic mutation of the germ-line 36-65 heavy chain gene can result in significant increases in affinity (12). They also provided hybridoma cells and monoclonal antibodies that could be used in reconstruction experiments to develop an assay for higher-binding mutants *in vitro*.

Reconstruction experiments were carried out with various ratios of 36-65 and each of the hybridomas producing higher-affinity antibodies. If we accepted a 50% or greater increase in the ELISA signal as marking a well with a possible higher-binding mutant, we could detect the presence of antibodies with the binding properties of 45-223 in the presence of a 250- to 500-fold excess of the lower-binding 36-65. Mutants with binding properties of 36-71 were de-

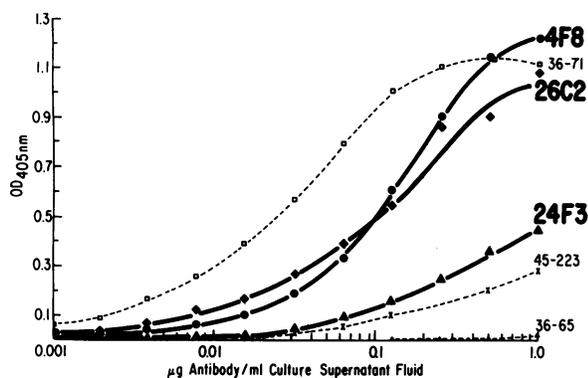


FIG. 1. ELISA analysis comparing the high-binding mutants to the parent and two high-affinity control monoclonals. The three representative mutants (4F8, 26C2, and 24F3) (bold lines) and the parent (36-65) ($2.5 \times 10^5 M^{-1}$) and two *in vivo* generated high-affinity control monoclonal antibodies [45-223 ($5.0 \times 10^6 M^{-1}$) and 36-71 ($4.5 \times 10^7 M^{-1}$)] (dotted lines) are shown. The concentration of each monoclonal antibody was quantitated from culture supernatants and assayed on a low-substituted Ars-albumin conjugate.

ected in the presence of an even larger excess of 36-65. These results suggested that we could detect mutants with a 20-fold or more increase in binding by plating out 250–500 cells in each well of a 96-well microtiter dish, allowing the cells to grow to confluence, and screening the medium for binding to the 0.4 mol of Ars per mol of albumin conjugate. If all of the wells from 20–30 plates were examined, $\approx 10^6$ cells could be screened for higher-binding mutants. Cells from positive wells would be plated at progressively lower densities and screened by ELISA to enrich for the presumptive mutants until they could be cloned. We have described a similar sort of “sib selection” assay for identifying hybridoma cells that have undergone class and subclass switching *in vitro* (32).

A number of sib selections were carried out with independent subclones of 36-65. Presumptive higher-binding mutants were identified at frequencies of $\approx 2.5\text{--}5 \times 10^{-5}$. Seven independent mutants were isolated. The binding curves of three representative mutant antibodies, 24F3, 26C2, and 4F8, are shown in Fig. 1. All three mutants bound antigen better than 36-65 and had binding curves that fell between those of the two higher-affinity control monoclonal antibodies, 45-223 and 36-71. Two of the mutants, 4F8 and 26C2, had binding curves similar to 36-71 and the third mutant, 24F3, had a binding curve similar to 45-223.

Characterization of the Mutants. The sequences of the heavy and light chain V regions of the three higher-binding mutants shown in Fig. 1 were determined by oligonucleotide-primed dideoxy nucleotide sequencing of the mRNA (40). The heavy and light chain V regions of 36-65 are identical to those reported (43, 44). The V_H region (Fig. 2A) contains the junctional serine at residue 95 required for Ars binding, whereas the V_L region (Fig. 2B) contains the junctional arginine at position 96 also required for Ars binding. As can be seen in Fig. 2, the heavy and light chain V regions of all three mutants had the exact same sequence as the 36-65 parental antibody. This surprising finding indicated that the large increases in binding of all three mutants were not due to amino acid changes in the V regions of these mutant antibodies.

To independently confirm this conclusion, we identified a switch variant (32) of 26C2 that had rearranged the 26C2 V region from the mutant $\gamma 1$ (26C2.G1) to a $\gamma 2b$ (26C2.G2b) C region. As can be seen in Fig. 3, the rearrangement of the 26C2 heavy chain V region to the downstream $\gamma 2b$ C region resulted in a reversion to wild-type binding. The sequence data (Fig. 2) and the genetic experiment shown in Fig. 3

suggested that the increase in binding was due to changes in the heavy chain C rather than the V region. This was confirmed when the mutant monoclonal antibodies were biosynthetically labeled and analyzed on a $NaDodSO_4$ /

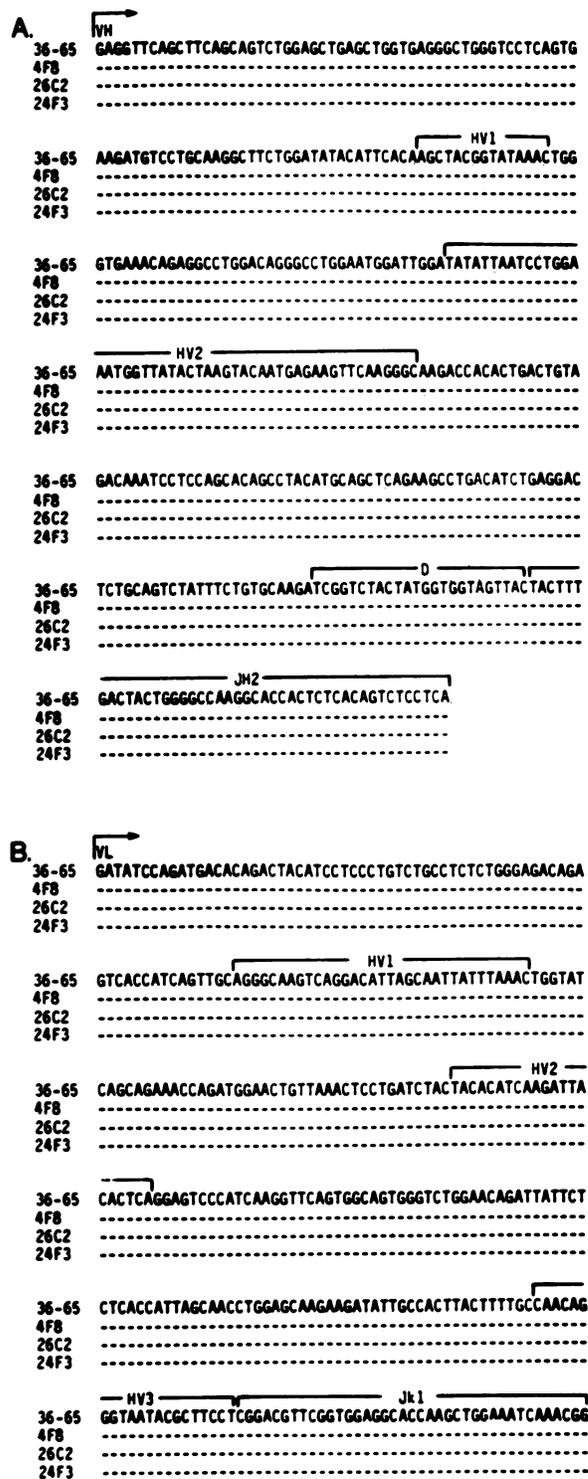


FIG. 2. Nucleotide sequence of the heavy and light chain V regions of the three increased antigen binding mutants compared to the germ-line sequence of the parent, 36-65. All gene sequences are derived from the A/J strain. The heavy chain V region sequence of 36-65 (A) is identical to the canonical $V_H Id^{CR}$, DFL16.1, and $J_H 2$ gene sequence (42), and the V_L region sequence of 36-65 (B) is identical to the consensus $V_{\kappa 10}$ -Ars-A and $J_{\kappa 1}$ sequences (43). Nucleotides are designated: A, adenosine; C, cytidine; G, guanosine; T, thymidine. A dash (–) means identical to the nucleotide in the line above.

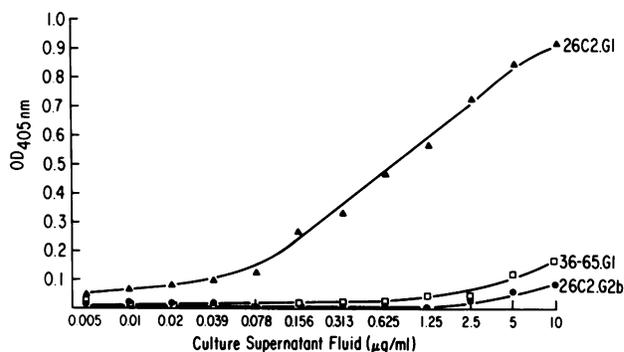


FIG. 3. ELISA analysis comparing the binding curves of a high-binding mutant to a spontaneous switch variant of the same cell line and to the parent monoclonal antibody, 36-65. The mutant (26C2) and parent (36-65) antibodies are of the IgG1 subclass (26C2.G1, 36-65.G1) and the switch variant of 26C2 is of the IgG2b subclass (26C2.G2b). The monoclonal antibodies were quantitated, assayed on a low-substituted Ars-albumin conjugate, and detected by using an alkaline phosphatase-labeled anti- κ chain reagent.

polyacrylamide gel (Fig. 4). These three mutants have three distinct phenotypes with respect to the size of their heavy chains: 26C2 has lost approximately one domain; 24F3 has lost part of a domain; and 4F8 is larger than the parental 36-65 heavy chain. These differences reflect the size of the heavy chain polypeptide since they persist when the proteins are biosynthetically labeled in the presence of tunicamycin in order to prevent N-linked glycosylation (data not shown). Furthermore, the polypeptide sizes have been confirmed by preliminary sequence data of the C regions (D.L.F., R.R.P., and M.D.S., unpublished data). The increase in binding is not due to changes in carbohydrate structure since 26C2 lacks the normal glycosylation site and does not contain any carbohydrate (data not shown). The multiple bands seen in 24F3 persist in the presence of tunicamycin and therefore are not due to glycosylation differences. Pulse-chase experiments suggest that the smaller bands are due to proteolytic degradation of the primary translation product.

Since the increased binding of these mutant proteins is associated with changes in the heavy chain C rather than the V region, it is likely that they reflected differences in avidity rather than true increases in affinity. In fact, binding assays with a low density of antigen are an excellent assay system for detecting increases in avidity. This was confirmed when we measured the association constants and found that the K_a values of these three mutants were indistinguishable from that of 36-65 (Table 1). The K_a determined here for 36-65 is well within the range of K_a values of $2.54 \times 10^5 \text{ M}^{-1}$ (12) and $4.1 \times 10^5 \text{ M}^{-1}$ (13) that have been published for this monoclonal antibody. We examined the fine specificity of binding of these mutants with analogues (35) of Ars and have not detected any differences between the mutants and 36-65 (data not shown). Since the mutants are also indistinguishable with a variety of monoclonal antiidiotype antibodies (data not shown), we have concluded that the conformation

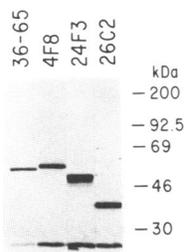


FIG. 4. NaDodSO₄/PAGE analysis of 36-65 and three mutants with increased antigen binding, 4F8, 24F3, and 26C2. All antibodies were biosynthetically labeled with [³⁵S]methionine, immunoprecipitated on Ars-Sepharose, eluted in 1% NaDodSO₄, reduced, and run on a Tris/glycine gel (10%).

Table 1. Affinity analysis of 36-65 and mutant cell lines

Cell line	K_a , M^{-1}
36-65	2.80×10^5
26C2	2.74×10^5
24F3	2.81×10^5
4F8	2.80×10^5

K_a values were determined by fluorescence quenching (12).

of the antigen binding site and of the V region is not changed due to the abnormality of the heavy chain C region.

DISCUSSION

We have described a combination of sib selection and an ELISA for identifying rare hybridoma subclones producing monoclonal antibodies that bind antigen better than the low-affinity antibody produced by the parental hybridoma. We have shown that these higher-binding mutants have heavy chain C rather than V region changes. Our results again raise the possibility that the inability to find V region mutants and the frequent and spontaneous occurrence of heavy chain C region mutants in hybridoma and most myeloma cells (19) reflects a relative stability of the V region gene compared to the C region gene in hybridoma cells. In fact, the real frequency of heavy chain C region mutation in the 36-65 cell line is probably much higher than 10^{-5} since our assay only detects those mutations that lead to a significant increase in binding. This suggests that mutations are occurring in the heavy chain C region at exceptionally high rates.

On the other hand, even with a positive identification system such as the one used here, it is unclear whether we would be likely to detect V region mutants even if they were occurring at a high rate. Studies on families of (4-hydroxy-3-nitrophenyl)acetyl (15) and oxazolone (16) binding monoclonal antibodies indicate that a single base change in the heavy chain hypervariable region can produce a 10-fold increase in affinity. We do not know if a single base change in 36-65 would give a detectable increase in binding since antibodies such as 45-223 and 36-71 have multiple amino acid substitutions. We also do not know whether changes in only one part of the V region would give a detectable increase or if amino acid substitutions in many different parts of the molecule would result in significant increases in affinity. These uncertainties make it impossible to propose even a rough estimate of the relative stability of the V and C regions in 36-65 and in other hybridomas and myelomas that have been studied and emphasize the need to develop better somatic cell genetic systems to examine this question.

Although we were unable to find V region mutants with higher binding, the ability of heavy chain C region changes to produce ≈ 100 -fold increases in binding could be very useful practically. We do not know the exact mechanism of this higher binding. However, it is clearly an increase in avidity rather than affinity. This increase could be due to increases in the flexibility of the short hinge region in IgG1. However, the results in Fig. 3 show that our assay does not distinguish binding differences between IgG1 and IgG2b, which has a longer and more flexible hinge region than IgG1 (45).

A second possibility is that the proteins are aggregating to produce polymeric antibodies. Preliminary experiments have shown that the mutant proteins are not aggregated in solution and that purified H₂L₂ monomers give the same higher-binding pattern. This suggests to us that the C regions are aggregating after binding to antigen, as has been reported for normal mouse IgG3 antibodies (46), resulting in an increase in the dissociation constant.

These observations indicate that it is possible to create higher-binding monoclonal antibodies by modifying the

structure of the C region. Further analysis of the C regions of these mutant proteins should reveal the changes that are responsible. With this knowledge, it may be possible to create cassettes with such C region changes, introduce any cloned heavy chain V region into the cassette, and transfect and express the engineered antibody in recipient cells. This would make it possible to improve the binding of many antibodies. It is possible that such antibodies will be especially useful for targeting tumors in situations where there are large amounts of circulating tumor antigens. The antibodies should rapidly dissociate from circulating antigens with one binding site but bind strongly to a solid tumor where multiple binding sites are presented, and the avidity of the antibody will diminish its dissociation.

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