In Vitro Affinity Maturation of a Specificity-Determining Region- Grafted Humanized Anticarcinoma Antibody: Isolation and Characterization of Minimally Immunogenic High-Affinity Variants

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ABSTRACT

Purpose: HuCC49V10 (V10), a humanized anticarcinoma monoclonal antibody (Ab) CC49, was generated by grafting only the specificity-determining regions (SDRs) of CC49 onto the variable light and variable heavy frameworks of the human Abs LEN and 21/28 CL, respectively. SDRs are those residues of the complementarity-determining regions that are most critical for antigen (Ag) binding. Compared with HuCC49, which was developed by conventional complementarity-determining region grafting, V10 has lower reactivity to the sera from patients who were previously given murine CC49 in clinical trials, although its Ag-binding affinity is 2–3-fold lower than that of HuCC49. To generate variants of V10 with higher Ag-binding affinity and lower sera reactivity, in vitro affinity maturation of V10 was carried out using phage display technique.

Experimental Design: A limited library of Fabs was generated by replacing some of the SDRs with all possible residues located at the corresponding positions in human Abs. The library was enriched, by several rounds of panning, in Fabs that have high affinity for the TAG-72 Ag. The clones encoding the best binders were expressed in insect cells as whole Abs that were purified and characterized.

Results: Competition radioimmunoassay and surface plasmon resonance measurements showed that two of the isolates, V14 and V15, have higher binding affinity than that of V10. In addition, the surface plasmon resonance analysis showed that the variants V14 and V15, compared with the parental V10, have lower reactivity to the anti-V region Abs using sera from patients who received murine CC49.

Conclusions: The two isolates, V14 and V15, which show higher Ag-binding reactivity and lower sera reactivity than the parental V10 Ab, are potentially more useful clinical reagents. These results demonstrate that phage display can be used to isolate variants of an Ab that are potentially less immunogenic in patients than the parental Ab from which they are derived.

INTRODUCTION

The clinical potential of antitumor mAbs5 is now being realized. Whereas most clinical studies using mAbs use chimeric or CDR-grafted (i.e., "humanized") forms of mAb, the possibility still exists that when these mAbs are administered to patients repeatedly, patients will mount anti-variable region responses to the murine residues residing in these chimeric or CDR-grafted immunoglobulin forms. This may be especially true in the use of radiolabeled mAb forms in which dose fractionation would be most favorable to limit radioisotope-mediated toxicity.

mAb CC49 recognizes tumor-associated glycoprotein TAG-72, which is expressed on the majority of colorectal, gastric, pancreatic, lung, and ovarian carcinomas (1–6). In athymic mice, this Ab efficiently targets and subsequently reduces or eliminates human colon carcinoma xenografts (7–14). Radiolabeled mCC49 has shown efficient tumor targeting of human colorectal (15–20), breast (21, 22), prostate (19, 23, 24), and ovarian carcinomas in Phase I/II clinical trials. Several ovarian cancer patients treated with 177Lu-labeled mCC49 were disease free for more than 18 months (25). Patients with metastatic breast and prostate cancer showed some partial responses after treatment with 131I-labeled mCC49 (22, 26). Not surprisingly,

5 The abbreviations used are: mAb, monoclonal antibody; anti-ID, idiotypic; HAMA, human antimurine antibody; BSM, bovine submaxillary mucin; CDR, complementarity-determining region; SDR, specificity-determining region; SPR, surface plasmon resonance; VL, variable light; VH, variable heavy; Ab, antibody; Ag, antigen; RIA, radioimmunoassay; mCC49, murine CC49; SB, Super Broth; pfu, plaque-forming unit(s); HuIgG, human IgG.
most patients given mCC49 showed HAMA responses. The results of the mCC49 clinical trials (17) also showed, however, that HAMA responses in a majority of the patients included anti-Id Abs.

In an attempt to avert HAMA and the anti-variable region responses it evokes in patients, CC49 was genetically engineered. Initially, the conventional approach to humanization was taken (27), and the CDRs of mAb CC49 were grafted onto the VL and VH frameworks of the human mAbs LEN and 21/28 CL, respectively, while retaining those residues of the murine frameworks that were deemed essential to the integrity of the Ag-binding site of the murine Ab (10). The resulting CDR-grafted humanized CC49 (HuCC49), although expected to be much less immunogenic than the murine Ab, could still evoke anti-V region, including anti-Id, responses mainly because of its murine CDRs. The potential immunogenicity of the murine CDRs of HuCC49 became more apparent when HuCC49 variants generated by replacing one or more murine CDRs with the corresponding CDRs of human mAbs were tested for their reactivity to the serum from a patient who had previously been given mCC49 in a clinical trial (17). Results of the analysis showed that whereas the LCDR3 of CC49 is most reactive with the patient’s anti-V region Abs, LCDR1 and HCDR2 are also moderately reactive (28). To minimize anti-V region responses, CC49 was humanized by grafting only the SDRs, the CDR residues that are most critical in Ag-Ab interaction. Compared with HuCC49, the reactivity of the resulting variant, HuCC49V10 (V10), to the anti-V region Abs in the sera of adenocarcinoma patients who were given 177Lu-labeled mCC49 in a Phase I clinical trial (17) was significantly lower. However, the relative Ag-binding affinity of V10 suffered a loss; it was about 3-fold less than that of HuCC49. One way of improving the clinical potential of the V10 is by maximizing its Ag-binding affinity. In vitro affinity maturation, using phage display technique, has been successfully used to improve the Ag-binding affinity of several Abs (29). However, some of the repertoire of mutations that an unrestricted phage display library is likely to generate could enhance the immunogenic potential of the Abs along with their Ag-binding affinity. To maximize the probability of generating only those mutations that may enhance the Ag-binding affinity, while reducing their immunogenicity in patients, a limited library of mutants could be generated by replacing some of the SDRs of V10 with all possible residues located at the corresponding positions in the human Abs.

The data presented in this study show that when a limited library of Fabs was generated, as described above, and enriched by several rounds of panning in isolates that have high binding affinity to the TAG-72 Ag, two of the isolates, HuCC49V14 (V14) and HuCC49V15 (V15), showed higher binding affinity than that of V10. More importantly, the reactivity of the variants and the parental Ab to the anti-V region Abs in the sera from patients in a Phase I clinical trial of mCC49 showed that V14 and V15 have much lower sera reactivity than that of V10. The two isolates, V14 and V15, which show significantly higher Ag-binding reactivity and much lower sera reactivity than the parental V10 Ab, have the potential to be much improved clinical reagents than the parental Ab they are derived from.

MATERIALS AND METHODS

Synthetic Oligonucleotides. The oligonucleotide primers used for DNA amplification were supplied by Biosynthesis Inc. (Lewisville, TX). The sequences of the primers used to generate DNA fragments for the L-chain library are as follows: 5’ V L (primer 1), 5’-TGACGGGACACAGACTGTGA-3’; 3’ V L (89) (primer 2), 5’-AGCTATATACGTCAT/G/C(A/T/C)(G/T)ACAATAATAG-3’; 3’ V H (91) (primer 3), 5’-GGGGATAGCTTAGT(G/C)(A/G/C/T)CTGCTGACA-3’; 3’ V H (92) (primer 4), 5’-TGAACGGGATAAAGTGTCAT/G/C(A/G/C/T)(A/G/ C/T)ATACTGCTGA-3’; 3’ V L (93) (primer 5), 5’-AGCT- GAGGGATAAAGTGTCAT/G(C)/A/G/C/T)(A/G/C/T)(A/ G/C/T)GCTATAATAC-3’; 3’ V L (94) (primer 6), 5’-CAAGCTG CAAAGCTT(G/C)(A/G/C/T)GGGATA-3’; 3’ V L (95) (primer 7), 5’-CAAGCTGGAGGATAAAGTGTCAT/G(C)/A/G/C/T)(A/G/ C/T)GCTATAATAC-3’; 3’ V L (96) (primer 8), 5’-GCCGCGCTGACTGCTTTGAGGCGAACTC-CCCTGTTGAGTCTTTTGAGGCGAACTC-3’.

The sequences of the primers used to generate the library of the Fd region of the H chains are as follows: 5’ V H (primer 9), 5’-GCCCCGTACATCCAGCCAGTTCCAGTGGTGA-3’; 3’ V H (50) (primer 10), 5’-CGGGAGAAACAGTGTCAT/G/C/T)TCCAATCCACT-3’; 3’ V H (52) (primer 11), 5’-CGTTTTCG GGGTACAGTGTCAT/G(C)/A/G/C/T)(A/G/C/T)(A/G/C/ T)GCTATAATAC-3’; 3’ V L (53) (primer 12), 5’-AATCTAGCTGTTGTCAT/G(C)/A/G/C/T)(A/G/C/T)(A/G/C/ T)GCTATAATAC-3’; 3’ V L (54) (primer 13), 5’-AAAAAT CATCGTACAGTGTCAT/G(C)/A/G/C/T)TCCAATCCAG-3’; 3’ V H (56) (primer 14), 5’-AGTCTACTTAAACAGTGTCAT/G(C)/A/G/C/T)TCCAATCCAG-3’; 3’ V H (58) (primer 15), 5’-TCTGTTGAGTACAGTGTCAT/G(C)/A/G/C/T)(A/G/C/T)(A/G/C/T)(A/G/C/T)GCTATAATAC-3’; and 3’ V H (primer 16), 5’-GCATGTAGCTTGTGACAAAGTACATTG-3’.

The designation of each of the mutagenic primers carries a parenthetically enclosed number, which indicates the position of the amino acid mutation induced by the primer. Each of the 5’ primers used for the first step PCR and the 3’ primers used for the second step PCR carries a unique restriction endonuclease site at its flank. The 5’ VL primer (primer 1) carries a SacI, whereas the 3’ VL (primer 8) has a XbaI site. The 5’ VH (primer 9) and the 3’ VH (primer 16) carry NcoI and SpeI sites, respectively. To eliminate an existing SacI site from the constant region of the κ chain, a point-mutated SacI site was incorporated into the 3’ VL primer (primer 8). The sequences recognized by the restriction endonucleases are italicized. Parenthetically enclosed nucleotides in the sequences indicate alternative nucleotides at that position in a mixture of mutagenic primers that were used.

DNA Amplification. Primer-induced mutagenesis was carried out by a dual-step PCR (30). The first PCR was carried out in a final volume of 50 μl, containing 10 ng of template, 200 μM deoxynucleotide triphosphates, and 5 units of Taq polymerase (Life Technologies, Inc., Gaithersburg, MD). The PCR mix contained 200 pmol each of the 5’ and 3’ primers (the latter being a mixture of mutagenic primers); primers 2–7 for the amplification of the L chain and primers 10–15 for the Fd region of the H chain. Mutagenic primers were mixed in a ratio that made all of the 3’ primers equimolar in concentration. Thirty cycles of a denaturing step at 94°C for 30 s, a primer annealing step at 55°C for 30 s, and a polymerization step at 72°C for 60 s were followed by a final primer extension step for
10 min at 72°C. The second PCR consisted of 30 cycles of denaturation (94°C for 30 s), primer annealing (55°C for 90 s), and polymerization (72°C for 90 s), followed by a final extension for 10 min at 72°C.

Phagemid Vector and Library Construction. A phagemid vector pComb3H-SS was used to generate a combinatorial library of the mutated Fabs displayed on the surface of the filamentous phage M13. pComb3H-SS, a modified version of the original pComb3 phagemid vector (31), was obtained from Dr. Carlos Barbosa of Scripps Research Institute (La Jolla, CA). The COOH-terminal part of gene III can be removed from the vector by cleavage with SpeI/NheI, followed by re-ligation. The 750-bp L chain PCR products were cloned between the SacI and XbaI sites of the pComb3H-SS vector. Ligation was performed, using a commercially available ligation kit (Life Technologies, Inc.) according to the conditions specified by the supplier. Electroporation-competent XL-1 Blue cells (Stratagene, La Jolla, CA), carrying a tetracycline resistance gene, were transformed with the ligated DNA by electroporation, using an electroporator (Bio-Rad, Hercules, CA) to supply a pulse of 1700 V at the field strength of 17 kV/cm for 5 ms. After adding SOC medium (Life Technologies, Inc.), the transformation mixture was streaked on the SacI and XbaI sites of the pComb3H-SS vector. Ligation was performed, using a commercially available ligation kit (Life Technologies, Inc.) according to the conditions specified by the supplier. Electroporation-competent XL-1 Blue cells (Stratagene, La Jolla, CA), carrying a tetracycline resistance gene, were transformed with the ligated DNA by electroporation, using an electroporator (Bio-Rad, Hercules, CA) to supply a pulse of 1700 V at the field strength of 17 kV/cm for 5 ms. After adding SOC medium (Life Technologies, Inc.), the transformation mix was incubated at 37°C for 1 h. Subsequently, SB (Life Technologies, Inc.) containing approximately 10^12 pfu was added to the culture and incubating for 1 h at 37°C, 1 ml of VCSM13 helper phage (Stratagene), which carries a kanamycin resistance gene, containing approximately 10^17 pfu was added to the culture. After incubation for 2 h, kanamycin (70 µg/ml) was added, and the culture was shaken overnight at 37°C. The phage was precipitated from the supernatant by adding PEG-8000 and NaCl to a final concentration of 4% and 3%, respectively, and incubating on ice for 30 min. After centrifugation, the pellet was suspended in 2 ml of TBS containing 1% BSA. The phage was titered by infecting XL-1 Blue cells (A_{600nm} = 0.5) with serial dilutions of phage suspension and plating the infected cells on LB/carbenicillin plates.

Selection of TAG-72-Binding Fabs by Panning. The phage library was screened and enriched for isolates binding to TAG-72. To that end, the library was subject to multiple (seven) rounds of panning. During each round, variants that specifically bind to TAG-72 were selected and amplified. For panning, a modification of the procedure that has been described previously (31, 32) was used. ELISA plates (Nalgene Nunc International, Rochester, NY) were coated overnight at 4°C with 50 µl of TAG-72-positive BSM (Type 1-S; Sigma, St. Louis, MO) in Dulbecco’s Phosphate Buffered Saline with calcium and magnesium chloride (Life Technologies, Inc.). The amount of BSM was progressively reduced from 1.0 µg/well to 0.01 µg/well, with increasing rounds of panning. The wells were washed with water and blocked by incubation with milk blocking solution (KPL, Gaithersburg, MD) at 37°C for 1 h. Fifty µl of phage (0.15–5 × 10^12 pfu) suspended in 50 µl of milk diluent solution (KPL) were preincubated at room temperature for 30 min before addition to the wells and incubated at 4°C overnight. After removing the milk/phage solution, the wells were washed by pipetting TBS/0.5% Tween 20 vigorously up and down. The washing cycles were progressively increased with increasing rounds of panning. Finally, the phage was eluted by adding 50 µl of elution buffer [0.1 M HCl (pH 2.2) and 1 mg/ml BSA] and incubating for 10 min at room temperature. The eluate was removed and neutralized with 43 µl of 1 M Tris base. The eluted phage was used to infect growing XL-1 Blue cells at room temperature for 15 min, and the virus was replicated in the presence of helper phage VCSM13. SB medium containing carbenicillin and tetracycline was added, and the culture was shaken at 37°C for 1 h. Phage preparations and pannings were repeated, as described earlier. After the final round of panning, the virus was harvested, precipitated and resuspended in TBS/1% BSA, as described previously (33).

Preparation of Soluble Fab: Genetic Manipulation of Phagemid for Soluble Fab Expression. The phage eluted from the last round of panning was used to infect logarithmically growing XL-1 Blue cells. The culture was grown overnight at 37°C in SB medium containing carbenicillin (20 µg/ml) and tetracycline (10 µg/ml). Cells were collected, and phagemid DNA was isolated and digested with NheI/SpeI. The large DNA fragment was self-ligated and used to transform competent XL-1 Blue cells. The transformation mixture was streaked on LB/carbenicillin plates, and the individual drug-resistant colonies were inoculated in 10 ml of SB medium containing 20 mM MgCl2 and 50 µg/ml carbenicillin. Cultures were grown at 37°C for 6 h before isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM for the induction of Fab expression. The culture was then shifted to 30°C and shaken overnight. Cells were pelleted, and a part of the cell pellet was saved to isolate the phagemid for the subsequent sequence analysis of the variants. The rest of the cell pellet was resuspended in 1 ml of PBS and lysed by four cycles of freezing and thawing. The cell debris was pelleted by centrifugation at 15,000 rpm, and the supernatant was collected for the Fab assay.

Screening of Fabs for Their TAG-72 Binding Affinity. The expressed Fab molecules were screened for their immunoreactivity to the TAG-72-positive BSM by SPR measurement, using BIAcore X instrument (BIAcore, Piscataway, NJ). All samples were run in duplicate over a sensor chip with 500 resonance units of BSA on another flow cell (flow rate 20 µl/min). The dissociation was observed for 300 s. After the samples were washed with the running buffer, the surfaces were regenerated with 1 M 3-(cyclohexylamino)-1-propanesulfonic acid buffer. The BIAeval 3.0.2 program was used to analyze the data. The BSA sensogram was subtracted from the corresponding BSM sensogram, and the Langmuir dissociation model was used to evaluate the off rate [k_{off} (36)].
Insect Cell Vectors and Generation of the H- and L-Chain Expression Constructs. Variants were expressed in insect cells as whole Abs, rather than as Fab fragments. To that end, expression constructs of the genes encoding the H and L chains of the variants were made in two different vectors, pIIZ/V5-His and pIB/V5-His (Invitrogen, Carlsbad, CA). One vector, pIIZ/V5-His, carries the zeocin resistance gene, whereas the other, pIB/V5-His, carries the blasticidin resistance gene. These genes are used for selecting stable transfectants of the insect cell line. The L-chain variants were cloned in the pIIZ/V5-His vector downstream from the OpIE2 promoter at the HindIII/Xhol site. The H chain expression constructs were made in pIB/V5-His vector. The insert was cloned at the HindIII/Xhol site, downstream from the OpIE2 promoter in pIB/V5-His vector.

Production of the Variant Abs and Their Immunoreactivity. To develop transfectomas secreting the HuCC49 variants, serum-free-adapted SF9 insect cells (Life Technologies, Inc.) were cultured in serum-free insect cell medium, SF-900 II SFM (Life Technologies, Inc.). Two million insect cells plated for 1 h at 37°C were cotransfected with 10 μg each of the pIIZ/V5-His-L chain and pIB/V5-His-H chain constructs, using Insectin-Plus Liposomes (Invitrogen) to mediate the transfections. After 4 days, the culture supernatants were harvested and tested, by ELISA, for immunoglobulin secretion and the reactivity of the secreted Ab to TAG-72. Stable transfectomas were selected in SF-900 II SFM medium supplemented with 200 μg/ml zeocin and 50 μg/ml blasticidin. The same selection medium was used to propagate the selected clones in suspension cultures.

ELISA. The ELISA assay for monitoring immunoglobulin production was carried out by a procedure described previously (37). To test the reactivity of the secreted Ab to TAG-72, 1 μg/well BSM was coated on the individual wells of the 96-well polystyrene microtiter plates. After saturation of the plates for 1 h at 37°C with milk blocking solution (KPL), 50 μl of diluted culture supernatants were added to the wells, in duplicate, followed by incubation at 37°C for 1 h. After a cycle of washings with the washing solution (KPL), 100 μl of peroxidase-conjugated anti-human IgG (Fcγ fragment specific), diluted 1:3000 in milk diluent solution (KPL), were added to the plates, and the incubation was continued for an additional hour at 37°C. The plates were washed before the addition of 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (KPL). The colorimetric reaction proceeded for 10 min at room temperature before the addition of the stop solution (KPL). The absorbance was read at 450 nm.

Purification of the Variant Abs and Their SDS-PAGE Analysis. The supernatants collected from the cultures of the transfectomas producing the variant Abs were centrifuged at 2000 × g for 10 min to remove cellular debris and loaded on a protein G-agarose column (Life Technologies, Inc.). Proteins bound to the column were eluted using 0.1 M glycine hydrochloride (pH 2.5). The pH of the eluted material was immediately adjusted to 7.4 with 1.0 M Tris (pH 8.0). The eluted proteins were concentrated using a Centricron 30 filter (Amicon, Beverly, MA) and dialyzed in PBS buffer using a Slide-A-Lyzer cassette (Pierce, Rockford, IL). The protein concentration was determined by the method of Lowry et al. (38). The purity of the eluted proteins was evaluated by SDS-PAGE, under reducing and nonreducing conditions, using pre-cast 4–20% Tris-glycine gel (Novex, San Diego, CA) and Coomassie Blue staining (Novex) visualization.

Competition RIA. The relative Ag-binding affinity of the variant Abs was determined using competition RIA, as described previously (28, 39). HuCC49 and mCC49 were included as positive controls, whereas HuIgG was used as a negative control in the assay. Twenty-five μl of serial dilutions of the Abs, resuspended in 1% BSA in PBS, were added to microtiter plates containing 10 ng of BSM saturated with 5% BSA in PBS. 125I-labeled HuCC49 (100,000 cpm in 25 μl of 1% BSA in PBS) was then added to each well. The assay was set up in triplicate. After an overnight incubation at 4°C, the plates were washed, and the bound radioactivity per well was counted in a gamma scintillation counter, and the values for Kd were calculated, using a modification of the Scatchard method (40). Also, the competition profiles were used to calculate the amount of each variant required to obtain 50% inhibition of the maximal binding of the radiolabeled HuCC49 to the BSM.

Flow Cytometric Analysis. Flow cytometric analysis was used to measure the binding of the variants to the TAG-72 expressed on the cell surface of a T-cell line, Jurkat. The procedure for fluorescence-activated cell-sorting analysis has been described previously (41). Different concentrations of Abs were used to compare their binding to the Jurkat cells expressing the cell surface TAG-72. The isotype-matched Ab, HuIgG, was used as a negative control, and V10 was included as positive control. To evaluate the ability of the variants to bind to cell surface TAG-72, 1 × 106 Jurkat cells were resuspended in cold Ca2+/Mg2+-free Dulbecco’s Phosphate Buffered Saline and incubated with the Ab to be tested for 30 min on ice. After one washing cycle, the cell suspension was stained with FITC-conjugated mouse antihuman Ab (PharMingen, San Diego, CA) for 30 min on ice. A second washing cycle was performed before the samples were analyzed with a FACScan (Becton Dickinson, Mountain View, CA) using CellQuest for Macintosh. Data from the analysis of 10,000 cells were obtained.

Immunoadsorption of Patients’ Sera. To assess the potential immunogenicity in patients, the HuCC49V10 variants were tested for their reactivity to sera stored from the adeno-carcinoma patients in a Phase I clinical trial (17). Patients in this clinical trial were given 125I-labeled mCC49 and found to have anti-V region Abs, including anti-Id Abs, to CC49 (28, 39). The sera of patients from the Phase I clinical trial were used to compare the reactivity of the variants to that of the parental V10. The sera, however, contain circulating TAG-72 Ag and antimurine Fc Abs that might interfere with the binding of HuCC49 and its variants to the sera anti-V region Abs. To overcome this difficulty, TAG-72 and Abs to murine Fc were removed from the sera by immunoadsorption before testing the sera reactivity. The procedure for immunoadsorption has been described previously (39). Essentially, CC92, a murine Ab that has the same isotype as CC49 and reacts with an epitope of TAG-72 distinct from the one recognized by CC49 (42), was coupled to Reactigel (HW65F; Pierce; Ref. 43). Serum was added to an equivalent volume of the CC92 gel (wet-packed volume) and incubated overnight at 4°C with end-over-end rotation. The
samples were centrifuged at 1000 × g for 5 min, and the supernatants were saved and stored.

**Evaluation of the Sera Reactivity of Variants Using SPR-Based Competition Assay.** The SPR-based competition assay that was used to test sera reactivities of Abs has been described previously (44). Essentially, SPR measurements were done with a BIACore X instrument using carboxymethylated dextran chips CM5 (BIACore). Proteins were immobilized on the CM5 chips by amine coupling (34). Competition experiments were done at 25°C on a sensor chip containing HuCC49 in flow cell 1 and rabbit γ-globulin (Bio-Rad) in flow cell 2 as a reference. A recently developed sample application technique (45) was used that made it possible to use small sample volumes. The binding was measured for 1000 s. After the unbound samples were removed from the surfaces by washing with running buffer using a flow rate of 100 μl/min, the surfaces were regenerated with a 1-min injection of 10 mM glycine (pH 2.0). The percentage of binding at each Ab concentration was calculated as follows: % binding = [slope of the signal obtained with competitor (serum + Ab)/slope of the signal obtained without competitor (serum only)] × 100.

**RESULTS**

**Generation of a Library of Variant Fabs of V10 mAb and Its Enrichment for High-Affinity Binders.** V10, a variant of HuCC49, was developed by grafting only the SDRs of CC49 onto human Ab frameworks. Although it is only minimally reactive with the anti-V region Abs of sera from patients who were treated with the mCC49, it has 2-fold lower Ag-binding affinity than that of HuCC49. To maximize the clinical utility of V10, in vitro affinity maturation was carried out to generate new isolates with higher affinity and preferably even lower sera reactivity than that of the parental V10. To that end, a limited library of isolates was generated by mutating some of the residues of LCDR3 and HCDR2, the two CDRs of CC49 that were previously shown to be important for Ag-binding activity and shown to be targets of the patient’s anti-V region response (28). The CDRs that previously showed no reactivity to patients’ sera were not genetically manipulated, lest such manipulation generate new idiotopes that become the new targets of the patients’ immune response. Only those residues of the LCDR3 and HCDR2 were mutated that were probably exposed and could contribute to the interaction with the Ag. Some exposed residues, which were deemed crucial in maintaining the structure of the combining site, were not changed. Not all amino acid residue types were used for replacement at the various positions. Only those amino acids that have been observed at those positions in human rearranged sequences were tried (Table 1). As described in “Materials and Methods,” mutagenic primers were designed, and the primer-induced mutagenesis was carried out by dual-step PCR amplification (30). A phagemid vector pComb3H-SS (31) was used to generate a combinatorial library of the mutated V10 Fabs. The phage library of ~ 2 × 10¹⁵, generated from ~ 1 × 10⁶ transformants, was enriched for isolates binding to TAG-72 by seven rounds of panning against TAG-72-positive BSM. The stringent conditions of panning (decreasing amounts of BSM on the plates and increasing number of washing cycles with the higher rounds of panning) reduced the titer of the phage recovered from the seventh round to ~ 1.2 × 10⁶ pfu. To use as control, a phage stock carrying the parental V10 Fabs was similarly prepared.

**Production of Soluble Fabs and Screening for High Binding Affinity Variants.** The DNA encoding the phage eluted from the last round of panning was genetically manipulated en masse to enable it to express soluble Fabs that can be subsequently screened for the high-affinity binders. The XL-1 Blue cells were infected with the phage, and the phagemid DNA was isolated from the transformed cells. After removing the DNA fragment encoding gene III, the phagemid DNA was self-ligated and introduced into XL-1 Blue cells, as detailed in “Materials and Methods.” Supernatants from the 48 cultures of the individual transformants were tested for the presence of Fab by ELISA and Western blot analysis. Of the 48 culture supernatants that were tested in duplicate by ELISA, a great majority of them were positive, and many among them were strongly positive (data not shown). Western blots that were done after electrophoresis under the nonreducing conditions showed a band of ~55 kDa (data not shown), a size in conformity with that of a Fab molecule. Similarly, a Western blot done after SDS-PAGE under reducing conditions yielded a band of 27–28 kDa (data not shown), a size expected of the Fd fragment. The Fab could be detected in 47 of the 48 culture supernatants that were tested, although the degree of the intensity of the bands varied. The expressed Fab molecules were screened for their immunoreactivity to the TAG-72-positive BSM by SPR measurements. Supernatants from all 48 cell cultures were analyzed for their reactivity to BSM. Because the Fabs were not purified, they were evaluated for their dissociation rates only. Fabs derived from mCC49, V10, and HuIgG were included as controls. The dissociation rates of only four isolates were lower than that of V10 (data not shown). The four variants were characterized further because they were likely to have higher affinity for TAG-72.

The phagemids prepared from the cell pellets of the four isolates were used for the sequencing of the inserts encoding the variable regions of the L and H chains. DNA sequencing was

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<th>Positions mutated</th>
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<tr>
<td>LCDR3 89, 91</td>
<td>R, L, N, K, M, H, Q, I, S</td>
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<td>LCDR3 92, 93, 94, 96</td>
<td>S, Y, R, A, G, H, T, C, P, D, N</td>
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carried out by the method of dideoxy-mediated chain termination (46). Amino acid sequences deduced from the nucleotide sequences showed substitutions in LCDR3 of all four variants. These substitutions were limited to positions 91 and 93, whereas no variant showed substitutions in HCDR2. Two variants showed inadvertent mutation in position 27b of the LCDR1 (Fig. 1).

Expression of V10 Variant mAbs in Insect Cells and Their Characterization. For further characterization of the variants, they were expressed in insect cells as whole Abs rather than as Fab fragments. To that end, sequences encoding each of the V region of the L-chain variants and those encoding the κ-chain constant region were assembled, along with the signal peptide sequence, in a vector containing a promoter that is functional in insect cells. Because none of the H-chain variants showed any mutations in HCDR2, the gene encoding V8, the H chain of the V10 (39) was paired with each of the L-chain variants for the production of the whole variant Ab. Accordingly, the sequence encoding V8, along with its signal peptide sequence, was cloned downstream from an insect cell-specific promoter in another vector. The expression constructs were introduced into Sf9 insect cells. When culture supernatants harvested 4 days after the transfection were tested by ELISA, transfectomas derived from all four variants, like those generated by the control construct of V10, were found to be producing whole IgG molecules. When the culture supernatants were assayed for their reactivity to TAG-72, the parental Ab V10 and the variants derived from it were found to be specific to TAG-72.

Variant Abs were produced and purified from the scaled up cultures of the highest producing clone of each variant. The concentrations of the secreted variant Abs in the culture supernatant were found to be within a range of 2–5 μg/ml. The purity of the proteins was evaluated by SDS-PAGE, under reducing and nonreducing conditions. Under nonreducing condition (Fig. 2A), a protein band of approximately 160 kDa was seen; the minor band may represent the break down product or may be due to incomplete immunoglobulin assembly. The reducing condition (Fig. 2B) yielded two protein bands of approximately 50–55 and 25–28 kDa, the sizes expected of the H and L chains of an IgG molecule.

Relative Ag-Binding Affinities of Variant mAbs Derived from V10. The relative Ag-binding affinities of the variant mAbs were determined by a solid-phase competition RIA, as described in “Materials and Methods.” HuCC49 and mCC49 were used as positive controls, whereas HulG served as a negative control. Serial dilutions of the purified unlabeled  

### Table: Mutations in V10 variants isolated by phage display.

<table>
<thead>
<tr>
<th>Variant</th>
<th>LCDR1</th>
<th>LCDR2</th>
<th>LCDR3</th>
</tr>
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<tbody>
<tr>
<td>V10</td>
<td>Val</td>
<td>Glu</td>
<td>Ser</td>
</tr>
<tr>
<td>V7</td>
<td>Leu</td>
<td>-</td>
<td>Lys</td>
</tr>
<tr>
<td>V12</td>
<td>-</td>
<td>Pro</td>
<td>Lys</td>
</tr>
<tr>
<td>V14</td>
<td>-</td>
<td>Pro</td>
<td>Ser</td>
</tr>
<tr>
<td>V15</td>
<td>Leu</td>
<td>-</td>
<td>Ser</td>
</tr>
</tbody>
</table>

Fig. 1 Mutations in V10 variants isolated by phage display. The SDRs in LCDR3 of mAb HuCC49 that were targeted for primer-induced mutations are shown in bold letters, whereas each of the other SDRs is marked with an asterisk. A non-SDR residue at position 97 that was already mutated to generate V10 is shown in italic. For V10, the entire amino acid sequence of the LCDR3 is shown, whereas for others, only the residues in the variants that replaced the residues of the parental V10 are shown. Only residue 27b of LCDR1, which was inadvertently mutated in two variants, is included in this figure. Dashes indicate the residues in variants that are identical to those of V10 at that position. The numbering convention of Kabat et al. (52) was followed.

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**Fig. 2** SDS-PAGE analysis of the purified mAb HuCC49 and its variants. Purified mAbs were analyzed under nonreducing (A) and reducing (B) conditions. Lane 1, HuCC49; Lanes 2–6, variants V10, V7, V12, V14, and V15. Sizes of the molecular mass markers (Bio-Rad) are given in the column to the left.

**Fig. 3** Competition RIA of mCC49-derived Abs. Increasing concentrations of mAbs mCC49 (●), HuCC49 (■), V10 (●), V7 (□), V12 (▲), V14 (△), V15 (○) and HuIgG (○) were used to compete for the binding of 125I-labeled HuCC49 to 10 ng of BSM coated in each well. Dashed line indicates competitor HuCC49. The assay was done in triplicate, and the error bars denote the SD from the mean value of the data in triplicate.
Abs (variant or control) were used to compete with radiolabeled HuCC49 for binding to the TAG-72-positive BSM. The results of the competition assay showed that all of the Abs, except the HuIgG control, were able to completely inhibit the binding of $^{125}$I-labeled HuCC49 to BSM. The competition profiles (Fig. 3) of the variants V7 and V12 were shifted to the right, whereas the profile of V14 was shifted only slightly, and that of V15 was shifted considerably to the left of the competition profile of the parental Ab V10. Competition profiles were used to calculate the amount of each unlabeled competitor required for 50% inhibition of the binding of $^{125}$I-labeled HuCC49 to BSM (Table 2). Compared with 90 ng of V10, 150 and 220 ng of variants V7 and V12, respectively, were needed. In contrast, 62 ng of V14 and only 30 ng of V15 were required for 50% inhibition of the binding of $^{125}$I-labeled HuCC49 to BSM. Thus, V15 is approximately 3-fold better than V10 in Ag binding. The relative affinity constants ($K_a$) of different Abs, presented in Table 2, were calculated from the competition experiments using a modification of the Scatchard method (40). The $K_a$ for V14 ($0.57 \times 10^8$ M$^{-1}$) was only comparable with that of V10 ($0.5 \times 10^8$ M$^{-1}$), whereas that of V15 ($1.16 \times 10^8$ M$^{-1}$) was significantly higher than that of V10.

Because of the complexity of the surface-binding reaction of whole Abs and the multiplicity of Ab-binding sites on BSM immobilized on a sensor chip, only a simple comparison of the binding signals at defined concentration of the Abs was semi-quantitatively carried out by SPR. This comparison was in agreement with the results of the relative affinities of V10, V14, and V15 as determined by the competition RIA (data not shown).

**Binding of the Variant mAbs to the TAG-72 Expressed on the Cell Surface.** Flow cytometric analysis was used to measure the binding of the V10 variants V14 and V15, to the TAG-72 expressed on the cell surface of a T-cell line, Jurkat. The procedure for fluorescence-activated cell-sorting analysis (41) has been detailed in “Materials and Methods.” In addition to the isotype-matched Ab, HuIgG, used as a negative control, V10 was included as a positive control. The data for the variant V15 are presented in Fig. 4; the binding profiles for V14 are not included for the sake of brevity. Two different amounts (0.5 and 0.25 μg) of each Ab were used to compare their binding to the Jurkat cells expressing cell surface TAG-72. When 0.5 μg of each Ab was used, the percentages of gated cells, calculated after exclusion of irrelevant binding, were 29%, 42%, and 46% for V10, V14, and V15, respectively. Thus, the two variants show significantly better binding to the cells displaying TAG-72 on their surface. A similar pattern of results was obtained when 0.25 μg of each Ab was used (data not shown).

**Sera Reactivity of V10 Variants.** To assess the potential immunogenicity of the V10 variants in patients, they were tested for their reactivity to sera stored from the adenocarcinoma patients in a Phase I clinical trial (17). Patients in this clinical trial were given $^{177}$Lu-labeled mCC49 and found to have anti-V region, including anti-Id, Abs to CC49 (28, 39). Sera reactivity was determined by a highly sensitive SPR-based competition assay (44). This assay involves the use of a device (BIAcore X

![Fig. 4](image-url) Flow cytometric analysis of the binding of HuCC49 variants to cells expressing cell surface TAG-72. Binding profiles of 0.5 μg of V10 (dark gray line) and V15 (black line) to Jurkat cells expressing TAG-72 on their cell surface. Binding of irrelevant mAb, HuIgG, is shown as a light gray area and represents ~2% of the cell population.

### Table 2. Affinity constants of CC49 and its derivative humanized antibodies

<table>
<thead>
<tr>
<th>mAb designation</th>
<th>Manipulated CDR</th>
<th>Positions substituted</th>
<th>ng (50% inhibition binding)</th>
<th>$K_a$ ($\times 10^8$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCC49</td>
<td></td>
<td></td>
<td>22</td>
<td>1.95</td>
</tr>
<tr>
<td>HuCC49</td>
<td></td>
<td></td>
<td>52</td>
<td>0.86</td>
</tr>
<tr>
<td>V10</td>
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</tr>
<tr>
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<td>0.36</td>
</tr>
<tr>
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<td>LCDR3</td>
<td>93</td>
<td>220</td>
<td>0.22</td>
</tr>
<tr>
<td>V14</td>
<td>LCDR3</td>
<td>91</td>
<td>62</td>
<td>0.57</td>
</tr>
<tr>
<td>V15</td>
<td>LCDR1/LCDR3</td>
<td>27b/91</td>
<td>30</td>
<td>1.16</td>
</tr>
</tbody>
</table>

*a* Numbering convention of Kabat et al. (52).
instrument) that monitors binding of the sera anti-Id (or anti-V region) Abs to HuCC49, and the inhibition of this binding by the variants. Fig. 5 shows a subset of sensorgrams generated by the inhibition of the binding of the anti-variable region Abs in serum of patient EA to HuCC49 immobilized on the sensor chip.

![Sensorgrams generated by competition SPR assay of the binding of patient’s serum to immobilized HuCC49. Increasing concentrations of V10 (A) and V15 (B) were used to compete for binding of anti-variable region Abs in serum of patient EA to HuCC49 immobilized on the sensor chip.](image)

**DISCUSSION**

This study describes the use of phage display, a powerful technique for increasing an Ab’s binding affinity (29), to max-

![Competition profiles developed by SPR-based assay of HuCC49 and its variants for binding of patients’ sera to immobilized HuCC49. Increasing concentrations of HuCC49 (●), V10 (□), V14 (▲), and V15 (○) were used to compete with sera from patients DS (A) and EA (B) to HuCC49 immobilized on the sensor chip. Percentage of binding of the sera to the immobilized HuCC49 was calculated from the sensorogram and plotted as a function of the concentration of the competitor.](image)
imize the Ag-binding affinity of the minimally immunogenic variant of HuCC49, V10. As in the process of affinity maturation during an immune response, where the repertoire of B cells is enriched in those cells displaying high-affinity Abs, in vitro affinity maturation through phage display is achieved by the enrichment of phages displaying higher affinity Fabs in the library. Accordingly, to enhance the affinity of V10, a repertoire of mutated Fabs derived from V10 were expressed and displayed on the surface of filamentous phage. Phages displaying mutated Fabs with higher Ag-binding affinity were selected by panning against TAG-72-positive BSM. However, unlike the process of somatic hypermutation in which affinities are built up through rounds of mutations and selection, in vitro affinity maturation requires all of the mutations to be present in the initial repository of mutants that is subjected to the rounds of selection. Therefore, to enhance the Ag-binding affinity of V10 while minimizing its immunogenicity in patients, a library of isolates was generated by mutating a limited number of residues that constitute the combining site and determine the specificity of an Ab and its affinity for the Ag. The Ab residues, which are primarily responsible for the binding properties of the molecule, are necessarily those that are located on the surface; the other residues mainly play an ancillary (structural) role.

The identity of the residues that interact with the Ag, the SDRs (47), can only be known from a determination of the three-dimensional structure of the Ab-Ag complex. In the absence of a three-dimensional structure, the identity of the SDRs could be guessed by a variety of ways. (a) An “alanine scan” could be performed, whereby every residue in the CDRs is systematically replaced by alanine, and then the effect of the replacement on the binding properties of the molecule is assessed. (b) The sequence of the mature Ab could be compared with that of the germ-line rearranged product (rearranged but before somatic hypermutation). However, in view of junctional diversity and the frequent addition and deletion of nucleotides during recombination, this is essentially an impossible task, except in the regions coded by the V and J gene segments. (c) The residue positions that display high variability could be identified with the SDRs (47). (d) The SDRs of other Abs, identified from Ab-Ag complexes of known structure (48), could be used as a first guess.

No three-dimensional structural information is currently available for CC49 or any of its variants. The putative CC49 SDRs, which were studied here, were chosen on the basis of known three-dimensional structures. The template for the mutational analysis is V10, which has been shown to have reduced immunogenicity and the same specificity as the original molecule, albeit with lower affinity (39, 49). In V10, the LCDR1 and LCDR2 of CC49 had been replaced by the corresponding regions of the human Ab LEN. Furthermore, only two murine residues in LCDR3 were kept; the rest were replaced with LEN residues. Furthermore, it was found that LCDR3, which is crucial for Ag binding, was not a factor in the immunogenicity of the molecule (28). In addition, no residues in LCDR1 were mutated. This is a short CDR, and several of its residues are often involved in the interaction with ligand. Furthermore, LCDR1 residues have been found to greatly influence the specificity of Abs. Moreover, LCDR1 was found not to be a target of the patient’s immune response in a clinical trial of mCC49 in adenocarcinoma patients (28). To ensure that the specificity of the CC49 variants will be preserved, no residue in LCDR1 was altered.

Because the objective of this study was to improve the affinity while reducing the potential immunogenicity of V10 in patients, only those residues that were probably exposed were mutated; the exposed residues could contribute to the interaction with Ag as well as act as targets for the patient’s immune response. Some exposed residues, which were deemed crucial in maintaining the structure of the combining site, were not changed. The V10 residues, which were subjected to mutational analysis, are presented in Table 1 and Fig. 2. Not all amino acid types were used for replacement at the various positions. Only those amino acids that have been observed at those positions in human rearranged sequences were tried (Table 1).

The following V10 residues were not mutated. (a) Residue 90 of L chain, a residue that is buried in Abs of known structure. (b) Residue 95 of L chain, a proline frequently found in κ chains, which has been found to be a cis-proline in known structures; the particular configuration of this cis-proline facilitates the formation of the LCDR3 loop. (c) Residue 51 of H chain, predicted to be a buried residue on the basis of known structures. (d) Residue 52a of H chain, a proline that has been found to be involved in a tight turn in Abs of known structure. Proline residues impose severe restrictions on backbone conformation, and the replacement of a proline with other amino acid types could cause a significant change in the structure and thereby the specificity and the affinity of the molecule. (e) Residue 55 of H chain, a residue that also has been found to be involved in a tight turn. (f) Residue 57 of H chain, a phenylalanine, which is expected to be buried on the basis of known structures.

Interestingly, some of the residues that were targets of mutation (residue 89, 91, 93, and 94 of L chain and residue 58 of the H chain) are encoded by codons that overlap the tetranucleotide A/G-C/T-A/T, which has been shown to be a mutational hot spot during in vivo affinity maturation (50). Also, it should be noted that some of the variants of V10 showed inadvertent mutations at position 27b of the L chain and position 62 of the H chain (data not shown). Such mutations might have been generated during several rounds of PCR that were carried out during the course of mutagenesis to generate these clones.

It should be noted that the parental Ab V10 has a relatively high affinity, and only a limited repository of mutant Fabs was derived from it and panned to select for high-affinity binders. It is not surprising, therefore, that Abs V14 and V15 showed only a modest increase in the Ag-binding affinity over the parental Ab V10. The affinity of V15, which showed 2–3-fold increase over V10, has become even better than that of HuCC49, the humanized CC49 from which the variant V10 was derived. The two variants, nevertheless, showed significantly better binding to the cells displaying TAG-72 on their surface. This modest improvement in the Ag-binding affinity was more than compensated by significantly lower reactivity of the isolates with sera from patients who were treated previously with mCC49. A Phase I pilot study in four patients with advanced carcinoma has recently been conducted (51) using a CH2-deleted variant of HuCC49, designated HuCC49ΔCH2 (11). Patients received a single infusion of ¹³¹I-labeled HuCC49ΔCH2. All patients had

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positive localization of Ab to metastatic tumor sites. The CH2-deleted construct had a more rapid clearance than mCC49. Three patients had no evidence of Ab response to HuCC49/CH2 over 6 weeks of observation, and one patient had a marginal response by week 6. Whereas these results are quite promising, it is still undetermined whether multiple dosing of this construct will lead to the appearance of anti-V region host responses. Furthermore, the genetic manipulations to develop the variants might have generated new epitopes to serve as targets for patients’ immune response. The studies reported here on the generation of both less immunogenic and higher affinity variants of HuCC49 may well provide an ultimately more suitable reagent for diagnostic or therapeutic applications.

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REFERENCES
In Vitro Affinity Maturation of a Specificity-Determining Region-Grafted Humanized Anticarcinoma Antibody: Isolation and Characterization of Minimally Immunogenic High-Affinity Variants


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