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Immunology

Humanized antibodies for antiviral therapy

(herpes simplex virus/computer modeling)

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ABSTRACT **Antibody therapy holds great promise for the treatment of cancer, autoimmune disorders, and viral infections. Murine monoclonal antibodies are relatively easy to produce but are severely restricted for therapeutic use by their immunogenicity in humans. Production of human monoclonal antibodies has been problematic. Humanized antibodies can be generated by introducing the six hypervariable regions from the heavy and light chains of a murine antibody into a human framework sequence and combining it with human constant regions. We humanized, with the aid of computer modeling, two murine monoclonal antibodies against herpes simplex virus gB and gD glycoproteins. The binding, virus neutralization, and cell protection results all indicate that both humanized antibodies have retained the binding activities and the biological properties of the murine monoclonal antibodies.**

It was first shown in 1891 that the antibodies induced during a viral infection can neutralize the inciting virus (1). For certain acute viral infections such as rabies, hyperimmune serum from infected patients has been a traditional therapy (2). More recently, the development of monoclonal antibody technology has allowed generation of specific antibodies against various viral antigens (3). Several reports have appeared showing that monoclonal antibodies can protect against various viral diseases in animal models (4–9). The use of monoclonal antibodies thus provides a new approach to antiviral therapy.

The production of murine monoclonal antibodies is relatively straightforward, but problems in the production of human monoclonal antibodies have persisted (10). In addition, the resulting human antibodies are frequently not of the appropriate isotype or do not possess the desired specificity. On the other hand, because xenogeneic antibodies are highly immunogenic in humans, the potential use of murine monoclonal antibodies for human therapy is limited, especially when repeated administration is necessary. The immune response against a murine monoclonal antibody may potentially be reduced by transforming it into a chimeric antibody. Such antibodies combine the variable binding domain of a mouse antibody with human antibody constant domains (11, 12). However, in a study to evaluate the immunogenicity of chimeric antibodies, it was found that the anti-variable domain response was not attenuated in the chimeric antibody, demonstrating that foreign variable frameworks can be sufficient to lead to a strong anti-antibody response (13). Therefore, for therapeutic purposes it may be necessary to fully humanize a murine monoclonal antibody by reshaping both the variable and the constant domains to make them human-like.

Winter and colleagues (14) first successfully humanized both chains of a rat antibody, directed against human lymphocytes, by introducing the six hypervariable regions from the rat heavy- and light-chain variable regions into human variable region framework sequences. Recently, a humanized antibody that binds to the human interleukin 2 receptor (p55) has also been reported (15). However, generation of other fully humanized antibodies has proved unexpectedly difficult, because significant loss of binding affinity generally resulted from simple grafting of hypervariable regions, probably due to distortion of the complementarity-determining region (CDR) conformation by the human framework.

Herpes simplex virus (HSV) infections range from asymptomatic to life threatening (16). More than 50 HSV polypeptides have been identified in HSV-infected cells, including seven major cell-surface glycoproteins (17). The specific biologic functions of these glycoproteins are not well defined, although gB and gD have been shown to be associated with cell fusion activity (18, 19). gB and gD express both type-specific and type-common antigenic determinants. Many of the antibodies against gB and gD have shown high neutralizing activities *in vitro* and *in vivo* (20–24). Oakes and Lausch (20) demonstrated that monoclonal antibodies against gB and gE suppress replication of HSV-1 in trigeminal ganglia. Dix *et al.* (21) showed that anti-gC and -gD antibodies protect mice against acute virus-induced neurological disease. Whitley and colleagues (22–24) produced a panel of murine monoclonal antibodies against HSV-1 and showed that several of the antibodies protected mice against encephalitis and death following ocular inoculation with the virus. Clone Fd79 (anti-gB) prevented encephalitis even when immunization was delayed until 48 hr postinfection. Fd79 and Fd138-80 (anti-gD) significantly reduced the severity of epithelial keratitis and lowered the frequency of persistent viral infection in an outbred mouse model, suggesting potential therapeutic uses in humans. Because murine monoclonal antibodies are limited by their immunogenicity for human therapy, we chose to humanize these two antibodies. In this article, we describe the construction of humanized antibodies for Fd79 and Fd138-80. These humanized antibodies retain the binding affinities and biological properties of the murine antibodies.

MATERIALS AND METHODS

Reagents. Vero cells were obtained from American Type Culture Collection (CCL 81) and maintained in minimum essential medium with 10% fetal bovine serum and nonessential amino acids. HSV-1 [Δ305 mutant (F strain)] (25) was a gift of Ed Mocarski (Stanford University). All enzymes were obtained from New England Biolabs and all chemicals were from Sigma unless otherwise specified. Staphylococcal protein A-Sepharose CL-4B was from Pharmacia. ^{125}I was from Amersham. Immunostaining reagents were ordered from Tago.

Synthesis of Variable Domain Genes. The construction of variable domain genes for the humanized antibody heavy chain and light chain generally follows ref. 15. The nucleotide sequences were selected to encode the protein sequences of the humanized heavy and light chains, including signal peptides, generally utilizing codons found in the mouse se-

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Abbreviations: HSV, herpes simplex virus; CDR, complementarity

quence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. For each variable domain gene, two pairs of overlapping oligonucleotides on alternating strands were synthesized (380B DNA synthesizer; Applied Biosystems), which encompassed the entire coding sequences as well as the signal peptide and the splice donor signal. Each oligonucleotide was 110–140 bases long with a 15-base overlap. Double-stranded DNA fragments were synthesized with Klenow polymerase, digested with restriction enzymes, ligated to the pUC18 vector, and sequenced. The two fragments with the correct sequences were then ligated into the *Xba* I sites of expression vectors similar to those described in ref. 15.

Expression and Purification of Humanized Antibodies. For each humanized antibody constructed, the heavy-chain and light-chain plasmids were linearized at the *Bam*HI sites and transfected into Sp2/0 mouse myeloma cells by electroporation. Cells were selected for gpt expression. Clones were screened by assaying human antibody production in the culture supernatant by ELISA.

Antibodies from the best-producing clones were purified by passing tissue culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B. The bound antibodies were eluted with 0.2 M glycine·HCl (pH 3.0) and neutralized with 1 M Tris·HCl (pH 8.0). The buffer was exchanged into phosphate-buffered saline (PBS) by passing over a PD10 column (Pharmacia).

Fluorocytometric Analysis. Vero cells were infected with HSV-1 at 3 plaque-forming units (pfu) per cell overnight. Cells were trypsinized at 0.5 mg/ml for 1 min, washed extensively with PBS, and resuspended in FACS buffer (PBS/2% fetal calf serum/0.1% azide) at $\approx 5 \times 10^6$ cells per ml. One hundred microliters of cell suspension was transferred to a polystyrene tube and incubated with 100 ng of purified antibody on ice for 30 min. The cells were washed with FACS buffer and incubated with fluorescein isothiocyanate-labeled goat anti-human antibody on ice for another 30 min. The cells were washed again and resuspended in PBS/1% paraformaldehyde. Cells were analyzed on a FACScan (Becton Dickinson).

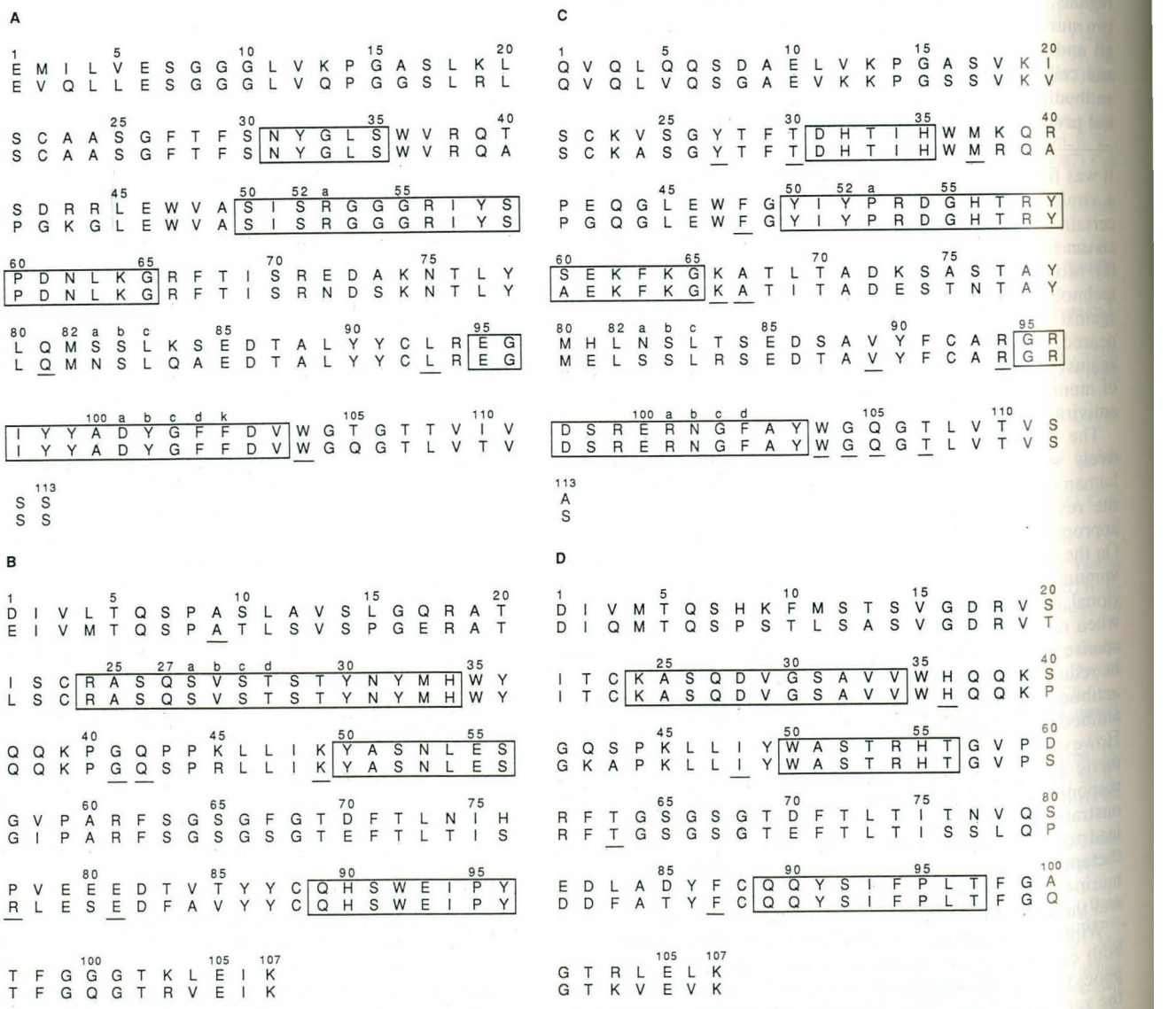

FIG. 1. Amino acid sequences of the heavy chain (*A*) and the light chain (*B*) of the murine and humanized Fd79 antibodies and the heavy chain (*C*) and light chain (*D*) of the murine and humanized Fd138-80 antibodies. The sequences of the murine antibodies as deduced from the cDNA (upper lines) are shown aligned with the humanized antibody sequences (lower lines). The humanized Fd79 and Fd138-80 framework sequences are derived from Pom and Eu antibodies, respectively. Residues are numbered according to the Kabat system (30). The three CDRs

Affinity Measurements. Binding affinities of the mouse and humanized antibodies were determined by competitive binding. Briefly, Vero cells infected with HSV-1 as described above were used as a source of gB and gD antigens. Increasing amounts of competitor antibody (mouse or humanized) were added to 1.5 ng of radioiodinated tracer mouse antibody (2 μCi/μg; 1 Ci = 37 GBq) and incubated with 4×10^5 infected Vero cells in 0.2 ml of binding buffer (PBS/2% fetal calf serum/0.1% azide) for 1 hr at 4°C. Cells were washed and pelleted, and their radioactivities were measured. The concentrations of bound and free tracer antibody were calculated. The binding affinities were calculated according to the methods of Berzofsky and Berkower (26).

Viral Neutralization Assay. Neutralizing activity of the murine and humanized antibodies was assayed by a plaque reduction method. Briefly, serial dilutions of antibodies were mixed with 100 pfu of virus and incubated at 37°C for 1 hr. The viruses were then inoculated onto six-well plates with confluent Vero cells and adsorbed at 37°C for 1 hr. Cells were overlaid with 1% agarose in complete medium and incubated for 4 days. Plaques were stained with neutral red. The antibody concentration was recorded for 90% plaque reduction.

***In Vitro* Protection Assay.** Twenty-four-well plates of confluent Vero cells were inoculated with virus at 0.1 pfu per cell and allowed to adsorb for 2 hr at 37°C before adding 1 ml of antibodies in medium (10, 1, or 0.1 μg/ml). At the end of 4 days, culture medium with antibodies was removed and plates were washed and dried by placing overnight in a 37°C incubator. To detect viral antigens, each well was incubated with 200 μl of mouse Fd79 antibody at 0.5 μg/ml for 1 hr at 37°C, washed twice, and incubated with 200 μl of peroxidase-conjugated goat anti-mouse immunoglobulin (1:300 dilution) for 1 hr at 37°C. The plates were washed and then developed with the substrate 3-amino-9-ethylcarbazole for 15 min at room temperature. The reaction was stopped by rinsing with water and air drying.

Computer Analysis. Sequence analyses and homology searches were performed with the MicroGenie sequence analysis software (Beckman). The molecular model of the variable domains was constructed with the ENCAD program (27) and examined with the MIDAS program (28) on an Iris 4D-120 graphics workstation (Silicon Graphics, Mountain View, CA).

RESULTS

Cloning of Heavy-Chain and Light-Chain cDNA. cDNAs for the heavy-chain and light-chain variable domain genes were cloned by using anchored polymerase chain reactions (29) with 3′ primers that hybridized to the constant regions and 5′ primers that hybridized to the dG tails (details to be published elsewhere). The heavy-chain variable domain gene of Fd79 belongs to mouse heavy-chain subgroup IIIB, and the light chain belongs to κ-chain subgroup III. The heavy chain and light chain of Fd138-80 belong to the heavy-chain subgroup II and κ-chain subgroup V, respectively. The translated amino acid sequences of the two antibodies are shown in Fig. 1.

Computer Modeling of Humanized Antibodies. To retain high binding affinity in the humanized antibodies, the general procedures of Queen *et al.* (15) were followed. First, a human antibody variable region with maximal homology to the mouse antibody is selected to provide the framework sequence for humanization of the mouse antibody. Normally the heavy chain and light chain from the same human antibody are chosen so as to reduce the possibility of incompatibility in the assembly of the two chains. Based on a sequence homology search against the NBRF protein se-

The computer program ENCAD (27) was used to construct a model of the Fd79 variable region. Inspection of the refined model of murine Fd79 revealed two amino acid residues in the framework that have significant contacts with the CDR residues (Table 1). Lysine in light chain position 49 has contacts with three amino acids in CDR2 of the light chain (L50Y, L53N, L55E; see Table 1 for explanation of coding system) and two amino acids in CDR3 of the heavy chain (H99D, H100Y). Leucine in heavy-chain position 93 shows interactions with an amino acid in CDR2 of the heavy chain (H35S) and an amino acid in CDR3 of the heavy chain (H100cF). Hence, L49K and H93L were retained in the construction of humanized Fd79, as their replacement with human Pom framework residues would be likely to introduce distortions into the CDRs. Also, seven other residues in the Pom framework (five in the light chain and two in the heavy chain) were substituted with consensus human residues (identical to the murine Fd79 sequence in six of the choices) because of their rare occurrence in other human antibodies. The elimination of unusual amino acids in the framework may further reduce immunogenicity. The murine Fd79 sequences and the corresponding humanized sequences are shown in Fig. 1 *A* and *B*. Substituted residues in the Pom framework are underlined.

Similarly, the murine heavy-chain and light-chain sequences of Fd138-80 were compared to the NBRF protein sequence data base, and the human antibody Eu was selected to provide the framework sequence for humanized Fd138-80. Inspection of a computer-generated model of Fd138-80 revealed six amino acid residues in the framework that show important contacts with CDR residues. The residues and their contacting counterparts are listed in Table 1; these murine residues were retained in the construction of humanized Fd138-80. Two other residues (L87F and H37M) show significant contacts with L98F, which is immediately adjacent to CDR3, so these two mouse residues were also retained. Eight amino acids in the Eu framework (two in the light chain and six in the heavy chain) were substituted with the murine residues (which are also consistent with the human consensus residues) because of their rare occurrence in other human antibodies. The murine Fd138-80 sequences and the corresponding humanized sequences are shown in Fig. 1 *C* and *D*. Substituted residues in the Eu framework are underlined.

Properties of Humanized Antibodies. The humanized Fd79 and Fd138-80 antibodies were characterized by comparisons with the murine and chimeric antibodies. Both humanized antibodies bind to Vero cells infected with HSV-1 in a fluorocytometric analysis in a manner similar to the chimeric

Table 1. Residues in the framework sequence showing contacts with residues in the CDRs

	Residue	Amino acid	Contacting CDR residues
Fd79	L49	K	L50Y, L53N, L55E, H99D, H100Y
	H93	L	H35S, H100cF
Fd138-80	L36	H	L34V, L89Q
	H27	Y	H32H, H34I
	H30	Y	H32H, H53R
	H48	F	H63F
	H66	K	H63F
	H67	A	H63F

The amino acid residues are numbered according to the Kabat system (30): the first letter (H or L) stands for the heavy chain or light chain, the following number is the residue number, and the last letter is the amino acid single-letter code. The CDRs are defined according

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