Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2869–2873, April 1991
Immunology

Humanized antibodies for antiviral therapy

(herpes simplex virus/computer modeling)

MAN SUNG CO*†, MARGUERITE DESCHAMPS*, RICHARD J. WHITLEY‡, AND CARY QUEEN*

*Protein Design Labs, Inc., 2375 Garcia Avenue, Mountain View, CA 94043; and ‡Department of Pediatrics, University of Alabama, Birmingham, AL 35294

Communicated by Stanley Falkow, January 14, 1991

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-

The publication costs of this article were defrayed in part by page charge

Abbreviations: HSV, herpes simplex virus; CDR, complementarity

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

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).

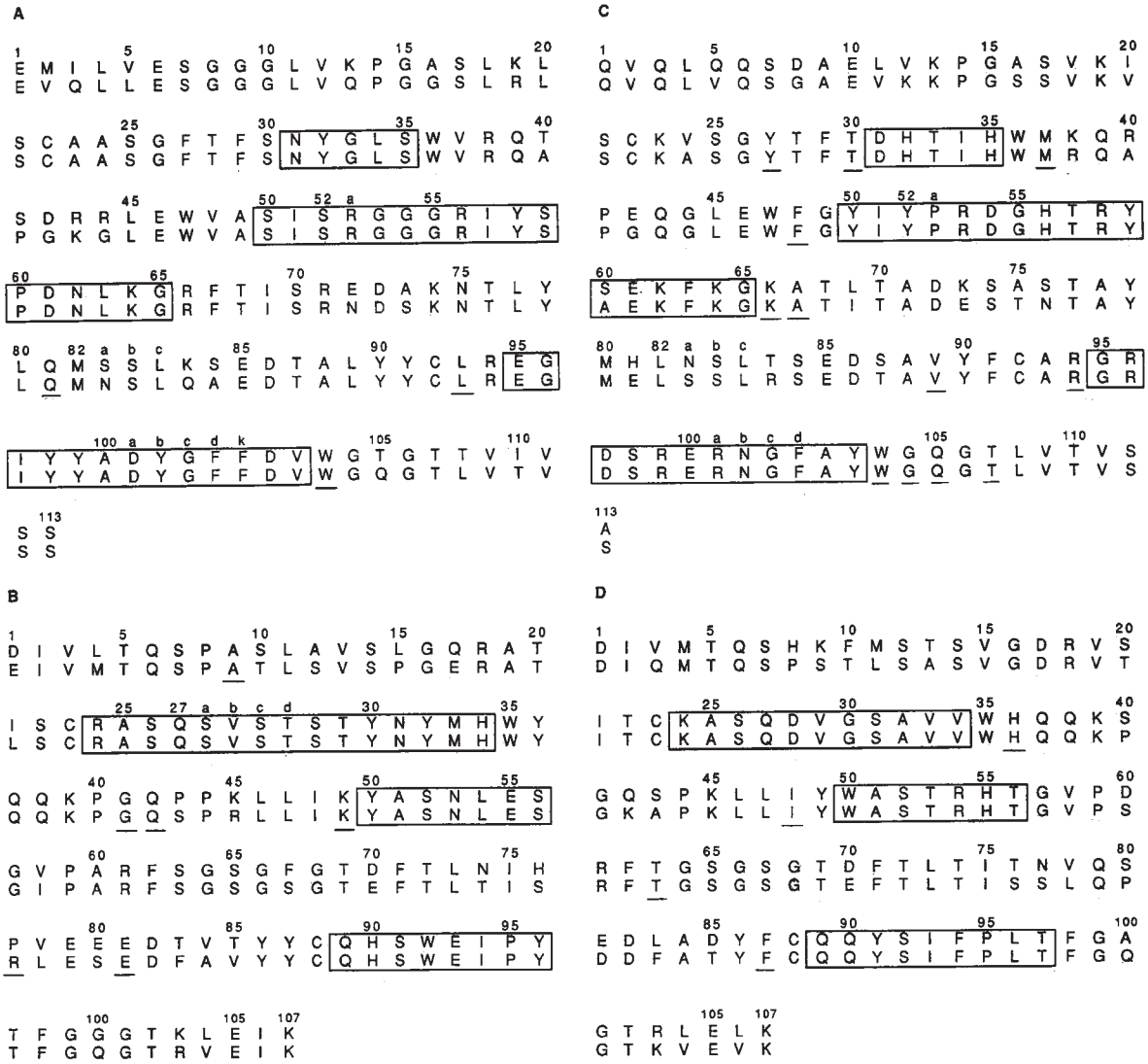


FIG. 1. Amino acid sequences of the heavy chain (*A*) and the light chain (*B*) of the murine and humanized Fd79 antibodies and the heavy chain (*C*) and light chain (*D*) of the murine and humanized Fd138-80 antibodies. The sequences of the murine antibodies as deduced from the cDNA (upper lines) are 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

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

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 to Kabat. Light chain: CDR1, residues 24–34; CDR2, residues 50–56;

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

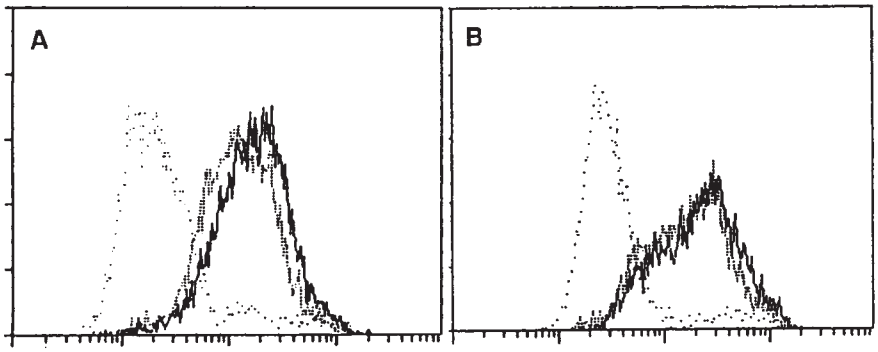


FIG. 2. Fluorocytometry of HSV-1-infected Vero cells stained with Fd79 (*A*) and Fd138-80 (*B*) antibodies. · · ·, Isotype-matched control antibody; ·····, humanized antibody; ——, chimeric antibody.

antibodies (Fig. 2), showing that they recognize their respective viral antigens. Chimeric antibodies (unpublished data) rather than the original mouse antibodies were used for this analysis so the same second-step staining reagent could be used. To more quantitatively assess the binding activity, radioiodinated murine antibodies were bound to virally infected cells and Scatchard analysis was performed. The affinities of the humanized antibodies were determined by competition with the iodinated antibodies. The measurements indicate that there is no significant loss of binding affinities in the humanized antibodies. Specifically, there is an ≈2-fold decrease in affinity in humanized Fd79 compared to the murine Fd79 (K_a, $5.3 \times 10^7\ M^{-1}$ vs $1.1 \times 10^8\ M^{-1}$). The affinity of humanized Fd138-80 is comparable to that of the murine antibody (K_a, $4.8 \times 10^7\ M^{-1}$ vs $5.2 \times 10^7\ M^{-1}$). These results suggest the general usefulness of computer modeling in the design of humanized antibodies.

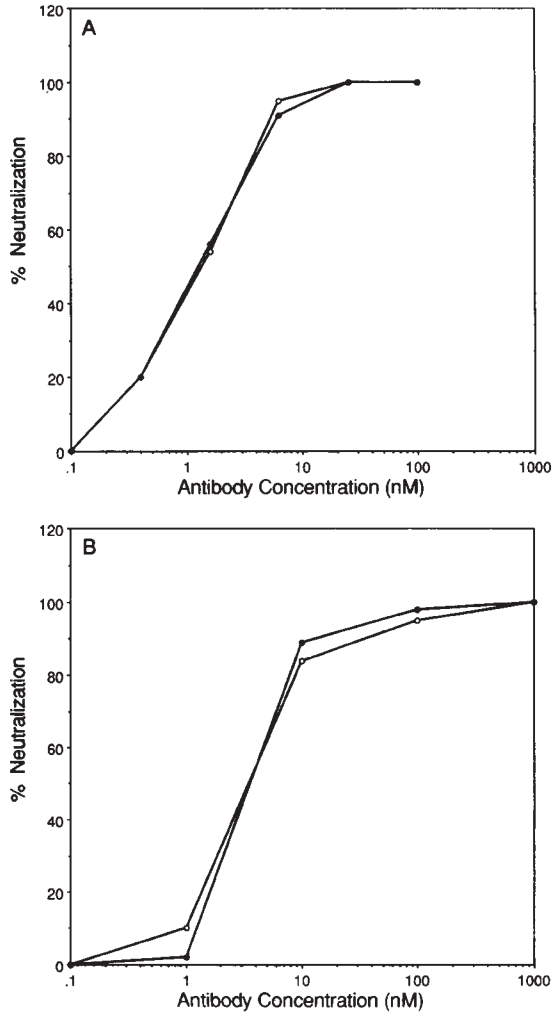
Murine Fd79 and Fd138-80 have been shown to neutralize HSV-1 *in vitro* without complement (22), so the neutralizing activities of the humanized antibodies were compared with the mouse antibodies. Serial dilutions of equal quantities of murine and humanized antibodies were incubated with virus for 1 hr before inoculation onto Vero cells. After 4 days, cells were stained with neutral red to visualize plaques. Results from these plaque-reduction assays indicated that both humanized Fd79 and Fd138-80 neutralize virus as efficiently as their murine counterparts (Fig. 3). Both humanized and murine Fd79 cause a 90% reduction of plaques at an antibody concentration of 7 nM (1 μg/ml). Similarly, humanized and murine Fd138-80 were able to cause a 90% plaque reduction at equivalent levels.

The antibodies were also investigated for their ability to protect cells from viral spread in tissue culture. Vero cells were inoculated with virus at 0.1 pfu per cell and allowed to adsorb for 2 hr at 37°C before addition of 10, 1, or 0.1 μg per ml of antibody. After 4 days, antibodies were removed and cells were stained with mouse Fd79 antibody for detection of viral antigens on infected cells. Results indicated that humanized Fd79 at 1 μg/ml (Fig. 4*A*) and murine Fd79 (data not shown) protected culture cells from viral spread. Cells stained with anti-gB antibodies were negative, except isolated single cells, which were infected with virus before introduction of protective antibodies. However, neither humanized (Fig. 4*B*) nor murine (data not shown) Fd138-80 was able to protect cells against viral spread, despite their ability to neutralize virus before inoculation. Fig. 4*B* shows that total cell lysis and staining with anti-gB antibodies were observed even in the presence of humanized Fd138-80 (10 μg/ml). Both gB and gD are thought to be associated with cell fusion and virus infectivity (18, 19). However, it is possible that Fd79 blocks both the infectivity and cell fusion functions of gB, while Fd138-80 does not block the fusion epitope of gD, so virus can still

that polyclonal antibody to glycoprotein D did not prevent the spread of virus from cell to cell in culture (31).

DISCUSSION

The binding, neutralization, and protection results all indicate that the humanized Fd79 and Fd138-80 antibodies have retained the binding activities and the biological properties of the murine monoclonal antibodies. The use of murine monoclonal antibodies for therapy is hindered by the generation in humans of an immune response to the mouse antibodies (32). The potential advantages of a humanized antibody are (*i*) the



DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

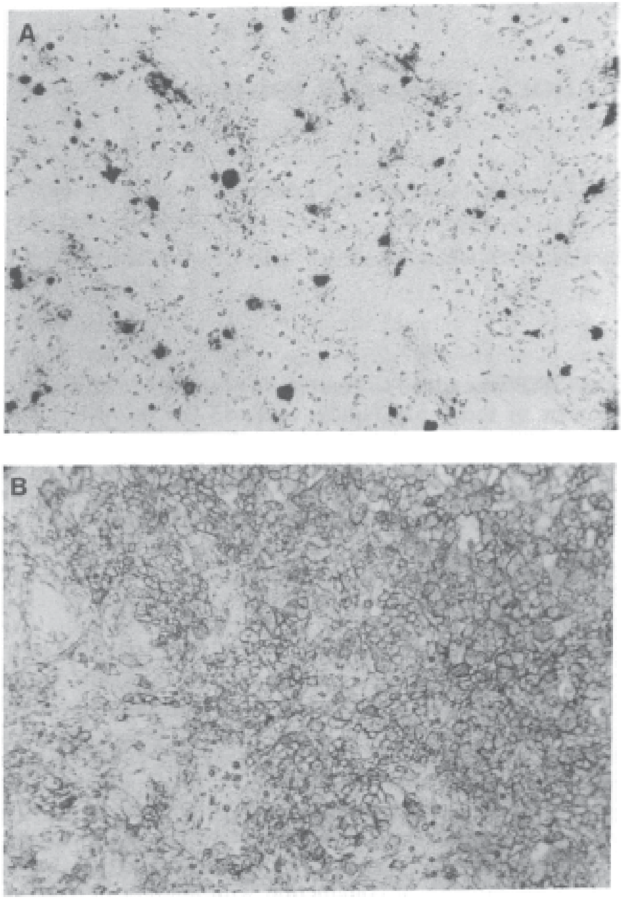


FIG. 4. Immunostaining of Vero cell monolayers infected with HSV-1 in the presence of humanized Fd79 antibodies (1 μg/ml) (*A*) and humanized Fd138-80 antibodies (10 μg/ml) (*B*).

lack of, or significantly reduced, immune response allowing repeated treatment; and (*ii*) an increased serum half-life, reducing the required dose as well as extending the effective period. It remains to be evaluated in clinical trials whether humanized antibodies will induce an anti-isotypic response or, more likely, an anti-idiotypic response, and whether humanized antibodies will be superior to chimeric antibodies. A humanized antibody (CAMPATH-1H) used to treat two patients with non-Hodgkin lymphoma was able to induce remission with no anti-globulin response (33).

The availability of humanized antibodies with specificity for HSV gB and gD should provide an opportunity for studying the *in vivo* potency and immunogenicity of humanized antibodies in treating viral diseases. The recognition by Fd79 and Fd138-80 of type-common epitopes of gB and gD (22) expands the therapeutic potential to HSV-2 as well as HSV-1. The use of a combination of two or more humanized antibodies in therapy could be important to reduce the development of antibody-resistant strains. Combination therapy of humanized antibodies with other antiviral agents such as acyclovir may provide further opportunities to combat diseases when chemotherapeutic agents alone have not been effective. The observation that Fd79 and Fd138-80 reduce the frequency of viral persistence in a murine ocular model (23) suggests that the humanized antibodies, perhaps together with other antiviral agents, could reduce episodes of recurrent genital infection, an area in which traditional antiviral agents have not been effective (34). The effector functions of the humanized antibodies remain to be studied. It is antici-
enhance effector functions such as antibody-dependent cellular cytotoxicity, leading to greater therapeutic efficiency in human patients. The actual efficacy of the antibodies in human patients must be evaluated by clinical trials.

We thank Michael Levitt and Phil Payne for helpful discussions in designing the humanized antibodies and Barry Selick for the expression vectors.

1. Babes, V. & Cerchez, T. (1891) *Clin. Bucuresci* **2,** 133.
2. Baltazard, M., Bahnanyan, M., Ghodssi, M., Sabeti, A., Gajdusek, C. & Rouzbehi, E. (1955) *Bull. WHO* **13,** 747–772.
3. Kohler, G. & Milstein, C. (1975) *Nature (London)* **256,** 495–497.
4. Schmaljohn, A. L., Johnson, E. D., Dalrymple, J. M. & Cole, G. A. (1982) *Nature (London)* **297,** 70–72.
5. Balachandran, N., Bacchetti, S. & Rawls, W. E. (1982) *Infect. Immun.* **37,** 1132–1137.
6. Rector, J. T., Lausch, A. R. N. & Oakes, J. E. (1982) *Infect. Immun.* **38,** 168–174.
7. Mathews, J. H. & Roehrig, J. T. (1982) *J. Immunol.* **129,** 2763–2767.
8. Letchworth, G. I. & Appleton, J. A. (1983) *Infect. Immun.* **39,** 208–212.
9. Kumel, G., Kaerner, H. C., Levine, M., Schroder, C. H. & Glorioso, J. C. (1985) *J. Virol.* **56,** 930–937.
10. James, K. & Bell, G. T. (1987) *J. Immunol. Methods* **100,** 5–40.
11. Morrison, S. L., Johnson, M. J., Herzenberg, L. A. & Oi, V. T. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6851–6855.
12. Boulianne, G. L., Hozumi, N. & Shulman, M. J. (1984) *Nature (London)* **312,** 643–646.
13. Bruggemann, M., Winter, G., Waldmann, H. & Neuberger, J. (1989) *J. Exp. Med.* **170,** 2153–2157.
14. Reichmann, L., Clark, M., Waldmann, G. & Winter, G. (1988) *Nature (London)* **332,** 323–327.
15. Queen, C., Schneider, W. P., Selick, H. E., Payne, P. W., Landolfi, N. F., Duncan, J. E., Avdalovic, N. M., Levitt, M., Junghans, R. P. & Waldmann, T. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 10029–10033.
16. Corey, L. & Spear, P. G. (1986) *N. Engl. J. Med.* **314,** 749–757.
17. Spear, P. G. (1985) in *The Herpesvirus*, ed. Roizman, B. (Plenum, New York), Vol. 3, pp. 315–356.
18. Cai, W., Gu, B. & Pearson, S. (1988) *J. Virol.* **62,** 2596–2604.
19. Fuller, A. O. & Spear, P. G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5454–5458.
20. Oakes, J. E. & Lausch, R. N. (1984) *J. Virol.* **51,** 656–661.
21. Dix, R. D., Pereira, L. & Baringer, J. R. (1981) *Infect. Immun.* **34,** 192–199.
22. Koga, J., Chatterjee, S. & Whitley, R. J. (1986) *Virology* **151,** 385–389.
23. Metcalf, J. F., Koga, J., Chatterjee, S. & Whitley, R. J. (1987) *Curr. Eye Res.* **6,** 173–177.
24. Metcalf, J. F., Chatterjee, S., Koga, J. & Whitley, R. J. (1988) *Intervirology* **29,** 39–49.
25. Post, L. E., Mackem, S. & Roizman, B. (1981) *Cell* **24,** 555–565.
26. Berzofsky, J. A. & Berkower, I. J. (1984) in *Fundamental Immunology*, ed. Paul, W. E. (Raven, New York), pp. 595–644.
27. Levitt, M. (1983) *J. Mol. Biol.* **168,** 595–617.
28. Ferrin, T. E., Huang, C. C., Jarvis, L. E. & Langridge, R. (1988) *J. Mol. Graphics* **6,** 13–17.
29. Loh, E. Y., Elliot, S., Cwirla, S., Lanier, L. L. & Davis, M. M. (1989) *Science* **243,** 217–220.
30. Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M. & Gottesman, K. S. (1987) *Sequences of Proteins of Immunological Interest* (Natl. Inst. Health, Bethesda, MD).
31. Hoggan, M. D., Roizman, B. & Turner, T. B. (1960) *J. Immunol.* **84,** 152–159.
32. Schroff, R. W., Foon, K. A., Beatty, S. M., Oldham, R. K. & Morgan, A. C. (1985) *Cancer Res.* **45,** 879–885.
33. Hale, G., Dyer, M. J. S., Clark, M. R., Phillips, J. M., Marcus, R., Reichmann, L., Winter, G. & Waldman, H. (1988) *Lancet* **ii,** 1394–1399.
34. Corey, L., Nahmias, A. J., Guinan, M. E., Benedetti, J. K., Critchlow, C. W. & Holmes, C. K. (1982) *N. Engl. J. Med.* **306,**

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.