Humanized Anti-Lewis Y Antibodies: \textit{In Vitro} Properties and Pharmacokinetics in Rhesus Monkeys

Man Sung Co, Jeanne Baker, K. Bednarik, E. Janzek, W. Neruda, P. Mayer, R. Plot, B. Stumper, Max Vasquez, Cary Queen, and Hans Loibner

ABSTRACT

ABL 364 is a murine monoclonal IgG3 antibody directed against the Lewis Y carbohydrate antigen (Leg) expressed on the surface of many epithelial cell tumors. The antibody mediates cytotoxicity via activation of human complement or human effector cells, and has been evaluated in several clinical trials including two Phase I/II trials in relapsed small cell lung cancer and metastatic breast cancer. To improve the effector functions of the antibody, increase its half-life in circulation, and avoid the human antimouse antibody response, two chimeric and several humanized antibodies were constructed for evaluation. The chimeric IgG1 is more potent than the murine IgG3 in tumor cell lysis via activation of human peripheral mononuclear cells (10-fold), but somewhat less effective in complement-dependent lysis (2-3-fold). The chimeric IgG3 is slightly less potent than the IgG1. A humanized IgG1 was constructed by combining the complementarity-determining regions of the ABL 364 antibody with human framework and constant regions. Several additional variants were subsequently constructed to improve the binding affinity and increase expression of the antibody. Two of the variants, designated I and K, differ by a single amino acid at position 75 of the heavy chain. Both variants have affinity within 2-fold of the chimeric IgG1 antibody and retain the cytolytic activities toward tumor cell lines. However, it was possible to express variant K at a significantly higher level (5-10-fold) than variant I. Pharmacokinetics of the humanized ABL 364 antibody variant K was compared with that of the parent murine antibody in rhesus monkeys. It was shown that the terminal half-life of the humanized antibody in rhesus monkeys is 14–20 days, with a mean of 16.3 days, while that of the parent murine antibody is only 1.9 days.

INTRODUCTION

A blood group-related carbohydrate, the Y difucosylated hapten (the Lewis Y antigen), has been found to be associated with 60–90% of human carcinomas of epithelial cell origin including breast, colon, gastric, and lung cancers (1, 2). The level of expression of the antigen has also been shown to correlate with survival in patients with carcinoma of the lung (3). Several murine antibodies have been generated against this antigen to explore their antitumor activities (1, 4, 5). The monoclonal antibody ABL 364 (formerly designated BR55–2 in previous literature) is a murine IgG3 that is highly specific for the Lewis Y carbohydrate antigen, and has shown significant \textit{in vitro} and \textit{in vivo} antitumor cytolytic activity (1, 2, 6, 7). The antibody activates both human complement and human effector cells \textit{in vitro} for tumor cell lysis (6, 7), and has been tested in several clinical trials including two Phase I/II trials in relapsed small cell lung cancer and metastatic breast cancer (8, 9). In the latter trial, it was shown that i.v. administration of murine ABL 364 can substantially reduce or eliminate Y antigen-positive cytokeratin-positive cells (“micrometastases”) in the bone marrow of patients with breast carcinoma (9). There are, however, several drawbacks in using murine antibodies for therapeutic purposes in humans. First, such antibodies usually induce a HAMA\textsuperscript{2} response (10, 11) that can render them ineffective for repeat therapy. Second, the half-life of murine antibodies in the circulation is relatively short (1–2 days) compared to human immunoglobulins (2–3 weeks; Refs. 12 and 13). Third, the Fc portion of murine antibodies may not elicit CDC or ADCC as effectively as the Fc portion of a human antibody (14, 15). To address these problems, recombinant DNA technologies have been applied initially to develop mouse/human chimeric antibodies (16) containing the variable region of the murine antibody and the constant region of a human antibody. However, chimeric antibodies may still provoke a substantial HAMA response because one third of the molecule is still of murine origin (17). To reduce further the immunogenicity of chimeric antibodies and to prolong half-life, humanized antibodies have been constructed by combining only the smallest required part of a mouse antibody, the CDRs, with human variable region frameworks and constant regions (18).

In this study, we initially constructed two chimeric antibodies of human IgG1 and IgG3 isotypes from the murine ABL 364 and evaluated their abilities to mediate CDC and ADCC against several human tumor cell lines, including breast, colon, gastric, and lung, in comparison to the parent murine IgG3. We then constructed a series of humanized antibody variants of IgG1 isotype and studied their binding affinities and effector functions. While studying the variants, we observed that a single amino acid substitution in the variable domain of heavy chain has a significant effect in determining how well the antibody can be expressed. The best humanized ABL 364 antibody variant has high affinity and specificity for the Lewis Y antigen and retains potent cytolytic activities toward several tumor cell lines, and can be expressed at a high level. Pharmacokinetics of this humanized antibody was evaluated in rhesus monkeys in comparison to the murine antibody.

MATERIALS AND METHODS

Cell Lines. ABL 364 (BR55–2) is secreted by a hybridoma generated by fusion of splenocytes of a BALB/c mouse immunized using the MCF7 breast carcinoma cell line with the 653 variant of the P3X63Ag8 murine myeloma (1, 2). MCF7, SKBR3, and SW948 were obtained from American Type Culture Collection. SKBR5, CATO, and SW2 were obtained from the Wistar Institute (Philadelphia, PA).

Cloning of V Region cDNAs. The variable domain cDNAs of the light and heavy chains of murine ABL 364 was cloned by the anchored PCR method (19) using 3' primers that hybridized to the C regions and 5' primers that hybridized to the G-tails attached to the cDNA using terminal deoxynucleotidyl transferase. The sequences were determined using the dyeodeoxy termination method on an Applied Biosystems 373A automated sequencer.

Expression Vectors. Separate expression vectors were constructed containing human light and heavy chain constant regions: pVκ for the human κ light chain, pVg1 for the human γ1 heavy chain, and pVg3 for the human γ3 heavy chain. These expression vectors have been described in detail previously (19). For construction of chimeric and humanized antibodies, the variable

\textsuperscript{2} The abbreviations used are: HAMA, human antimouse antibody; CDC, complement-dependent cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; CDR, complementarity-determining region; PBMC, peripheral blood mononuclear cell; IC\textsubscript{50}, 50\% inhibitory concentration.

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\textsuperscript{1} To whom requests for reprints should be addressed, at Protein Design Labs, 2375 Garcia Avenue, Mountain View, CA 94043.
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region cDNAs of the murine or humanized antibodies can be inserted into the XbaI sites of the corresponding expression vectors (19).

Construction of Chimeric Light and Heavy Chain Expression Plasmids. To generate the variable region genes, primers were synthesized that annealed to the 5′ and 3′ ends of the cloned V_{L} or V_{H} cDNA of the murine antibody. The 5′ primers contained an XbaI site followed by a ribosome recognition signal (CCACC), an ATG initiation codon, and the next 15 nucleotides of the variable region signal peptide sequence. The 3′ primer contained the last 15 nucleotides of the variable region coding sequence, followed by a mouse J_{H}4 or J_{L}3 intronic segment to provide a splice signal, and then an XbaI site. The fragments generated by PCR with these primers were digested with XbaI and cloned into the XbaI site of the respective expression vectors. The correct orientation and sequence of the cloned segments in the plasmids were verified by sequencing.

Construction of Humanized Light and Heavy Chain Expression Plasmids. Nucleotide sequences were selected that encode the protein sequences of the humanized ABL 364 light and heavy chains, including signal peptides, generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or remove undesirable ones. For each variable region gene, two pairs of overlapping oligonucleotides on alternating strands were synthesized (Applied Biosystems 380B DNA synthesizer), which encompassed the entire coding sequences as well as a splice donor signal and contained suitable restriction sites at their ends. The oligonucleotides were 110-140 bases long with 15-base overlaps. Double-stranded DNA fragments were synthesized with Klenow polymerase from the 5′ pair and separately from the 3′ pair of oligonucleotides, digested with restriction enzymes, ligated into the pUC18 vector, and sequenced. A 5′ fragment and a 3′ fragment with correct sequences were then excised from pUC18 and ligated together into the XbaI sites of the expression vectors pVk, pVg1, or pVg3. To construct the heavy chain variants, oligonucleotides with the desired sequences were synthesized and incorporated into the variable region gene using the PCR.

Transfection of S194 Cells. Approximately 10^5 cells of the murine myeloma cell line S194 (ATCC TIB 19) were washed and resuspended in 10 ml DMEM + 50 mM Tris (pH 7.3) + 250 μg/ml DEAE-dextran containing 10 μg each of the appropriate light and heavy chain expression plasmids and incubated at 37°C for 1 h. Cells were then centrifuged and washed in serum-free medium and finally placed in 15 ml DMEM containing 10% horse serum. Supernatant was harvested 24 or 48 h later and assayed for the presence of antibodies using the ELISA.

Transfection of Sp2/0 Cells. Transfection of Sp2/0 cells (ATCC CRL 1581) was by electroporation using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) at 360 V and 25 μF capacitance according to the manufacturer's instructions. Before transfection, the light chain and heavy chain containing plasmids were linearized using BamHI, extracted with phenol-chloroform, and ethanol precipitated. All transfections were done using 20 μg plasmid DNA and about 10^7 cells in PBS. The cells from each transfection were plated into one 96-well tissue culture plate. After 48 h, selective medium was applied. Cells were selected in DMEM + 10% FBS + HT media supplement (Sigma, St. Louis, MO) + 1 μg/ml mycophenolic acid. After the wells had become confluent with surviving colonies of cells, medium from each well was assayed for the presence and quantity of secreted antibodies using the ELISA. A high-yielding clone from each transfection was sub cloned and expanded to produce antibody for purification and characterization.

Purification of Chimeric and Humanized Antibodies. Chimeric IgG1 and IgG3 antibodies were purified from culture medium using protein A-Sepharose and protein G-agarose chromatography, respectively. Humanized IgG1 variants were purified using protein A-Sepharose. The protein concentration was estimated by measuring the absorbance at 280 nm (assuming 1 mg/ml = 1.3 absorbance units). Antibodies were more than 95% pure when analyzed using SDS-PAGE.

ELISA for Determination of Antibody Concentration. Antibodies in the culture supernatants were quantitated with the ELISA using goat antihuman γ chain (Tago, Burlingame, CA) as the capture agent and horseradish peroxidase-conjugated goat antimouse IgG as the detection antibody. Standard curves were calibrated using purified human or humanized ABL 364. Relative Binding Affinities Using Flow Cytometry. Several concentrations of test antibodies were prepared by serial 2-fold dilutions. One hundred μl of the test antibodies were incubated with 100 μl SKBR3 cells (2 x 10^6/ml) on ice for 1 h in a buffer containing PBS + 2% fetal bovine serum + 0.1% sodium azide. After incubation, cells were washed, centrifuged, and resuspended in FITC-conjugated goat antimouse antibody and incubated for 30 min on ice. Finally, the cells were washed and then fixed in 200 μl PBS + 1% paraformaldehyde before analysis with flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). An antibody was considered to have X times the affinity of another antibody if only 1/X the concentration was required to produce equal mean fluorescence intensity. In our experience, relative binding affinities determined in this manner correlate very well with affinities determined by competitive binding.

Binding Affinity Measurements. SKBR3 cells were washed and resuspended in PBS + 1% BSA + 0.1% azide (binding buffer) to 2 x 10^6 cells/ml. The cell suspension (100 μl) was added to each well of a microtiter plate (Immunoplate II; Nunc, Naperville, IL). The plate was centrifuged, the supernatants removed, and the following antibody solutions added: 50 μl FITC-conjugated ABL 364 (4 μg/ml) and 50-μl dilutions of test antibodies from 2048 μg/ml to 1 μg/ml. All dilutions were in binding buffer. The plates were incubated overnight at 4°C. After centrifugation of the microtiter plate, the supernatants were transferred into another plate for determination of fluorescence in a fluorescence ELISA reader. The cell pellets were washed with 200 μl ice-cold FCS, and the supernatants were collected and measured as described above. The cell pellets were resuspended in 100 μl PBS/well and also measured in the fluorescence reader. The blank values were obtained by incubation of the cells with 100 μl binding buffer instead of antibody solutions and subtracted from the respective antibody values. The concentration of free antibody was calculated by adding the fluorescence units of the supernatants and the washing solutions. For determination of affinity constants, the ratio of bound/free fluorescence tracer (R) was plotted against the concentration of free competitor. The binding affinities were calculated according to the method of Benfolds and Berkower (20). Alternatively, antibodies were labeled with 125I using the Bolton-Hunter method (21), and radioactivity of supernatants and cell pellets was measured in a gamma counter.

CDC. 51Cr-labeled target cells were mixed with test antibodies and fresh serum (as a source of complement) and incubated overnight at 37°C. The supernatants were harvested with a harvesting press (Skatron) and counted in a gamma counter. For determination of total 51Cr release, human serum was replaced by 2% SDS. Spontaneous release was obtained by replacing human serum by medium and antibody by PBS. After counting, lysis was calculated by:

\[
\% lysis = \left( \frac{\text{total release} - \text{spontaneous release}}{\text{total release}} \right) \times 100
\]

ADCC. This assay was performed manner similar to the CDC assay described above. Instead of human serum, human PBMCs in the desired E:T ratio were used.

Pharmacokinetics in Rhesus Monkeys. Three monkeys in each group received a single dose i.v. bolus injection of 0.8 mg/kg body weight murine ABL 364 or humanized antibody variant K. Blood was withdrawn at appropriate time points, and antibody concentrations in sera were determined using the ELISA described below. The terminal elimination half-life was obtained from the log concentration versus time curve by least-square regression analysis. The ELISA for determination of the serum concentration of murine ABL 364 and humanized antibody variant K in rhesus monkey serum was based on a monoclonal anti-idiotypic antibody (22) directed against the antigen-binding site of the murine and humanized ABL 364 antibodies. The anti-idiotypic antibody was coated on microtiter plates. Test sera containing murine or humanized antibodies in appropriate dilutions were incubated for 60 min at 37°C. Samples containing murine antibody were developed with peroxidase-conjugated rabbit antimouse IgG; samples containing humanized antibody were developed with peroxidase-conjugated goat antihuman IgG. To generate standard curves, murine ABL 364 or its humanized variant were diluted in pure rhesus monkey serum. The sensitivity of this ELISA is approximately 1 ng/ml.

RESULTS

Cloning of V Region cDNAs. The murine ABL 364 antibody heavy and light chain V region cDNAs were cloned using an anchored PCR method (19). Several independent light chain clones were sequenced and found to be identical; similarly, several heavy chain

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clones had the same sequence. The nucleotide sequences of the light and heavy chain V regions including the signal peptides and the translated protein sequences are shown in Fig. 1. The CDRs of each chain are underlined. The V<sub>L</sub> domain belongs to the mouse κ chain group II and uses the J<sub>4-4</sub> segment. The V<sub>H</sub> domain belongs to the mouse heavy chain subgroup III(D) and uses the J<sub>4-3</sub> segment.

Construction of Chimeric Antibodies. PCR with appropriate primers was used to copy the V<sub>L</sub> region, including the signal sequence, from an ABL 364 light chain cDNA clone. The 5′ primer was designed to insert a ribosome recognition sequence before the ATG initiation codon, and the 3′ primer was designed to insert a splice donor signal after the J segment. The PCR-generated fragment was then cloned into the XbaI site of the pVgl expression vector (19), thus creating a complete chimeric κ chain gene with a mini-intron between the mouse V-J and human C<sub>r</sub> segments. Similarly, PCR was used to insert the ABL 364 V<sub>H</sub> region, including a ribosome recognition sequence and J segment splice donor signal, into the XbaI sites of the pVgL and pVg3 vectors (19). The resulting plasmids contain complete chimeric γ<sub>L</sub> and γ<sub>3</sub> heavy chain genes, respectively, with a mini-intron between the mouse V-D-J segment and the human C<sub>j1</sub> exon.

The chimeric light chain-containing plasmid was transfected into Sp2/0 mouse myeloma cells along with either the chimeric γ<sub>1</sub>- or chimeric γ<sub>3</sub>-containing plasmid, and cells were selected for expression of the gpt gene. Production of antibody by surviving clones of cells was determined using the ELISA. The best producing clones secreted 5 μg/ml chimeric IgG1 and 10 μg/ml chimeric IgG3/10<sup>6</sup> cells/24 h. Chimeric IgG1 and IgG3 antibodies secreted into the media were purified using protein A-Sepharose or protein G-agarose, respectively. The purified IgG1 and IgG3 chimeric antibodies were at least 95% pure when analyzed using SDS-PAGE (data not shown).

Effector Functions of Chimeric Antibodies. The activities of the chimeric antibodies against several Lewis Y antigen-positive human tumor cell lines (SKBR3, breast cancer; SW948, colon cancer; and CATO, gastric cancer) were evaluated in the presence of human complement (CDC) or human peripheral mononuclear cells (ADCC). The chimeric IgG1 is quite potent against the cells via CDC (IC<sub>50</sub> = 1–5 μg/ml), while the chimeric IgG3 is less potent. Surprisingly, the murine IgG3 is more potent in CDC than the chimeric IgG1 by 2–4-fold (Fig. 2). In contrast, the chimeric IgG1 is significantly more potent in ADCC with human PBMCs or monocytes than the murine IgG3 (by 10-fold), whereas the activity of chimeric IgG3 is similar to that of murine IgG3 (Fig. 2).

Construction of Humanized ABL 364 Antibody. To retain the binding affinity and specificity of the murine antibody in the humanized antibody, the general procedures of Queen et al. (23) were followed. First, human antibody light and heavy chain V regions with maximum sequence homology to the respective murine ABL 364 V regions were selected to provide the framework sequences for the humanized antibody variable domain to minimize the chance that the CDC conformation would be distorted when grafted onto the human framework. When humanizing previous antibodies, we chose the light chain and heavy chain from the same human antibody to reduce the possibility of incompatibility in the assembly of the two chains. However, based on a sequence homology search against the National Biomedical Foundation Protein Identification Resource, the Tew and Porn antibodies were selected to provide the framework sequences for the humanized ABL 364 light and heavy chain, respectively. Indeed, the ABL 364 light chain variable region shows a significantly higher homology to the Tew framework (77%) than to any other human light chain framework (in the range of 50%). Therefore, Tew was chosen to provide the framework for the humanized light chain variable region, despite the lack of availability of a sequence for the Tew heavy chain. Porn was chosen to provide the framework for the heavy chain because of its high homology to the ABL 364 heavy chain sequence.

Next, the computer programs ABMOD and ENCAD (24) were used to construct a molecular model of the ABL 364 variable domain. Inspection of the refined model of murine ABL 364 revealed several amino acid residues in the framework that may have significant contacts with the CDR residues and that differ between the mouse and human sequences. Specifically, residue L54 Ser in the light chain can interact with the CDRs. Residues H73 Asp, H74 Asn, and H109 Thr in the heavy chain also show significant potential interactions with the CDRs. These murine residues were therefore retained in the V region framework of the humanized ABL 364 antibody. Different human light and heavy chain variable regions exhibit substantial amino acid homology within the framework regions (25). However, a given variable region usually contains amino acids atypical of other variable human regions at several framework positions. The Tew light chain contains such unusual residues at position 108, and the Porn heavy chain at positions 82 and 87. At these positions, we chose to use a consensus human residue rather than the framework residue in the humanized antibody to further minimize potential immunogenicity.

As described in “Materials and Methods,” DNA sequences were synthesized that encoded the desired humanized ABL 364 light and heavy chain V regions along with signal peptides and splice donor.
sequences. The respective sequences were inserted into the XbaI sites of the pVκ and pVκ1 vectors to generate complete humanized κ and γ1 genes. The resulting expression plasmids were transiently transfected into S194 cells or stably transfected into Sp2/0 cells as appropriate.

Variants of Humanized ABL 364. As determined with flow cytometry ("Materials and Methods"), the humanized antibody showed a small but measurable 5-fold reduction in affinity compared to the chimeric antibody, and, perhaps more importantly, it was very poorly expressed (Table 1). Reexamination of the computer model of the variable domain and comparison of the murine and humanized antibody sequences revealed a potential problem at position 95 in the heavy chain. This residue does not contact the CDRs but is important in interfacing with the light chain. A heavy chain mutant was thus constructed to retain the residue 95 from the murine framework (variant D). Several additional variants were constructed by altering amino acids where there is charge change between the murine and humanized framework (variants E-G). Variants D and F improved the binding affinity by a factor of 2.5 and 2, respectively, when analyzed by flow cytometry, while the others had no effect (Table 1). Two additional variants (H and I) were constructed to combine improvements in earlier variants. The variant I, which incorporated three additional murine framework residues (residues 42, 44, and 95) from the initial version, showed an affinity within 2-fold of the chimeric IgG1.

However, all of the variants remained poorly expressed, when evaluated by transient expression in S194 cells or by stable expression in Sp2/0 cells. The expression levels were 5—10-fold lower when compared to the expression of chimeric IgG1. Several variants were constructed by replacing segments of the humanized heavy chain variable region with the analogous murine segments to identify the framework region responsible for the low level of expression. Then additional variants were constructed by substituting each differing residue of the segment with the murine residue and assayed by transient transfections of S194 cells. A new variant (K) was identified by incorporating the murine framework residue at position 75 into variant I. Variant K improved the expression level by 5-fold, to a level comparable to the chimeric antibody, and also retained binding affinity within 2-fold of the chimeric antibody (Table 1). The amino acid sequences of the humanized ABL 364 (variant K) light and heavy chain V regions are shown compared to the murine sequences in Fig. 3.

**Table 1 Properties of humanized ABL 364 heavy chain variants**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Residue substitution</th>
<th>Antibody expressed (μg/ml)</th>
<th>Relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimeric H</td>
<td>Murine V_H</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Pom H</td>
<td>Initial humanized V_H</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Variant D</td>
<td>Y95H</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>Variant E</td>
<td>G42E</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Variant F</td>
<td>G44R</td>
<td>0.15</td>
<td>0.4</td>
</tr>
<tr>
<td>Variant G</td>
<td>N84S, S85R</td>
<td>0.15</td>
<td>0.3</td>
</tr>
<tr>
<td>Variant H</td>
<td>Q3K</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Variant I</td>
<td>G42E, G44R, Y95H</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Variant K</td>
<td>G42E, G44R, Y95H, S75A</td>
<td>1.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Each humanized ABL 364 antibody variant was expressed by transfecting the ABL 364 humanized κ light chain and the respective heavy chain construct into S194 cells, and the supernatants were assayed using the ELISA. The relative affinities were measured by binding of the antibody variants to SKBR3 cells by flow cytometry (see "Materials and Methods").
3.0 × 10^7, 1.7 × 10^7, and 1.2 × 10^7 M^-1 for murine, chimeric, and humanized (variant K) ABL 364, respectively. Alternatively, affinities were also measured using iodinated antibodies, and similar results were obtained. In addition, binding to a synthetic Lewis Y covalently bound to BSA also showed comparable specific bindings (data not shown).

CDC. The activities of the humanized variant K against a panel of Lewis Y antigen-positive human tumor cell lines (SKBR5, breast cancer; SW948, colon cancer; SW2, small cell lung cancer; and MCF7, breast cancer) were evaluated in the presence of human complement (CDC). Results similar to those of chimeric IgG1 (Fig. 2) were obtained. The IC_{50} of the four experiments is shown in Fig. 5. The humanized antibody showed half-maximal killing at an average of 8 μg/ml versus 4.5 μg/ml for chimeric antibody. The slight loss of activity may reflect the slightly reduced affinity of the humanized antibody compared to the chimeric antibody. The murine antibody is slightly more potent (average IC_{50} at 2 μg/ml) than the chimeric antibody in CDC, probably a result of the cooperativity effects of the murine γ3 constant region (discussed below).

ADCC. The antibody-dependent cytotoxicity of the humanized ABL 364 (variant K) was also evaluated against the panel of four tumor cell lines. The IC_{50} of the four experiments is shown in Fig. 6 and remained consistent with the results for the chimeric IgG1 determined earlier (Fig. 2). Both humanized and chimeric antibodies (average IC_{50} at 1 and 0.5 μg/ml, respectively) were significantly better than the murine antibody (average IC_{50} at 8 μg/ml), presumably reflecting better interactions of human PBMCs with the human Fc than with the murine Fc.

Pharmacokinetics in Rhesus Monkeys. The pharmacokinetics of the humanized ABL 364 (variant K) in rhesus monkeys was compared with that of the parent murine antibody. Serum concentrations of both antibodies were determined by a selective and sensitive ELISA. This assay makes use of a highly specific reagent, a monoclonal anti-idiotypic antibody raised against the murine antibody that binds
activities, in comparison to the parent murine antibody, toward a panel of Lewis Y-positive cancer cells, including breast, colon, and gastric cancers. Both chimeric IgG1 and IgG3 bind competitively with the murine ABL 364 antibody to the Lewis Y antigen-positive cell lines. However, chimeric IgG1 lyses the cells via activation of human peripheral mononuclear cells approximately 10-fold more effectively than the chimeric IgG3, which has activity comparable to the murine antibody. This is consistent with the observation by Schreiber et al. (29) that the chimeric IgG1 of another Lewis Y antibody, BR96, showed stronger antitumor effects than the murine IgG3 in a nude mice model. Surprisingly, the murine antibody was more effective in mediating CDC than chimeric IgG1 (2-3-fold), which was in turn more effective than the chimeric IgG3. The mechanism of enhanced CDC by the murine IgG3 is not fully understood, although it has been speculated that intermolecular interactions between Fc regions of IgG3 molecules may lead to enhanced binding to multivalent antigens or multivalent interaction with C1q molecules (30). The difference in effectiveness of the antibodies on the various cell lines is probably due to differences in the expression of Lewis Y on the cell surface. Indeed, this observation has been reported by another murine IgG3 against the disialoganglioside GD3, which showed that the level of cell lysis mediated by this antibody is correlated with GD3 antigen density on the cell surface (31). Despite the significant toxicity of murine IgG3 toward many tumor cell lines, the chimeric IgG1 has proven to be more desirable, particularly with its more potent ADCC activities and the potential reduction of immunogenicity in humans.

To reduce further the potential immunogenicity of chimeric antibodies, humanized antibodies were constructed. Antibodies of IgG1 isotype were chosen to take advantage of its more potent effector functions. The humanization of antibodies is still not routine, with many investigators reporting significant loss of binding affinity when CDRs from a murine antibody are grafted into a human framework (32, 33). Our method of choosing a highly homologous human framework and then using computer modeling has often allowed us to humanize a murine antibody without significant loss of affinity in a single try (19, 23, 34, 35). However, in the case of ABL 364, the first humanized version generated had a small...
but measurable loss of affinity of about 5-fold. Using PCR to quickly create variants and a fast relative affinity measurement based on flow cytometry, we were able to rapidly generate and evaluate a series of antibody variants and obtain a humanized antibody which retained affinity to within 2-fold of the chimeric antibody. The observation that the murine antibody has a slightly higher apparent affinity than the chimeric antibody, despite having an identical variable domain, was intriguing. This can be due to a unique property of the murine IgG3 isotype, in which a noncovalent intermolecular interaction of Fc domains may lead to a higher binding avidity (30). The binding affinity of ABL364 to the Lewis Y antigen, on the order of $10^7$ M$^{-1}$, is common among antibodies generated against carbohydrate antigens (36, 37).

In the course of constructing the humanized ABL364 antibody, we also observed that substitution of a single amino acid in the heavy chain framework can significantly affect the ability of the antibody to be expressed. We demonstrated that changing residue 75 in the humanized heavy chain framework from Ser to Ala caused the antibody to be secreted at a 5—10-fold higher level when transiently expressed in S194 cells. Similar results were observed when a population of clones generated by stable Sp2/0 transfections were compared. The effect of this substitution was confirmed by the reverse experiment of mutating the murine framework residue 75 in the chimeric antibody from Ala to Ser and observing a corresponding decrease in expression of the antibody by transfected S194 cells (data not shown). It is unclear how residue 75 affects the ability of the antibody to be expressed, although previous publications have reported that a single amino acid substitution in the $\kappa$ light chain variable region can prevent secretion of an antibody (38—40). In one case, it was shown that the immunoglobulin chain was arrested in the endoplasmic reticulum (40).

Since Lewis Y antigen is expressed in a significant percentage of human carcinomas of epithelial cell origin including breast, colon, gastric, and lung cancers, there is considerable interest in its use as a target for antibody therapy or diagnosis. Several murine monoclonal antibodies have been reported reactive with this antigen (4, 5). Blaszczyk-Thurin et al. (1) reported generation of BR55—2 (subsequently renamed ABL364), a murine IgG3 highly specific for the Lewis Y antigen and very potent in in vitro cytotoxicity against a panel of Lewis Y-positive tumor cells (6, 7). Hellström and coworkers (4, 29) also showed that another murine IgG3, BR96, demonstrated significant antitumor activity against human lung carcinomas xenografted into nude mice. More recently, Kitamura et al. (5) reported construction of chimeric and humanized forms of another Lewis Y antibody 3S193 for evaluation. In addition, single-chain immunotoxin or doxorubicin immunoconjugate has been prepared and shown to be highly potent in the killing of tumor cells in vitro or in an athymic mice model (41, 42). One of the best characterized anti-Lewis Y antibodies is ABL364 (BR55—2). This antibody has been reported to react in vitro with 60—90% of the tumors of epithelial origin including colorectal (128/189), pancreas (26/28), breast (16/23), gastric (8/12), and lung (57/71) (2). In vitro, the antibody exhibits potent ADCC and CDC activities against many tumor cell lines (6, 7). More significantly, this antibody has been tested in five human clinical trials including two Phase I/II trials in relapsed small cell lung cancer and metastatic breast cancer (8, 9). However, the clinical utility of ABL364, like that of other murine antibodies, was limited by the HAMA response and its short half-life. We have therefore constructed a humanized ABL364 to fully utilize the potential of this antibody in human therapy. Indeed, recent clinical trials of two humanized antibodies against interleukin 2 receptor (43) and CD33 (44) for treatment of acute graft-versus-host disease and myeloid leukemia, respectively, have shown that these humanized antibodies did not develop any HAMA response in human patients.

The humanized ABL364 (variant K) retains the binding affinity of the murine antibody and is more effective in activation of human effector cells for tumor cell destruction. The pharmacokinetics of the humanized ABL 364 (variant K) in rhesus monkeys has also been studied and compared with that of the parent murine antibody. The $\beta$ half-life of the murine antibody is 1.9 days, similar to that observed in cancer patients (11, 12). In contrast, the $\beta$ half-life of humanized ABL364 is 14—20 days, similar to that of human IgG1 in humans (13, 45, 46). Moreover, the fact that a circulating level of humanized ABL364

Fig. 7. Pharmacokinetics profile in the rhesus monkey. Concentrations of murine ABL 364 (○, [A]) or humanized ABL 364 (variant K; O, □, △) in rhesus monkey serum were measured following an 0.8 mg/kg body weight single dose i.v. bolus injection. Blood was withdrawn at appropriate time points, and antibody concentrations in sera were determined using the ELISA.
was maintained in the monkeys for 90 days and accelerated clearance was not observed (Fig. 7) suggests that there was little or no monkey antibody response against humanized ABL 364. Based on these properties, the humanized ABL 364 may be a promising candidate for immunotherapy of certain tumors. The ability of the murine antibody to reduce micrometastatic cells disseminated to the bone marrow suggests that humanized ABL 364 may be most effective in a minimal residual disease setting to increase disease-free survival time or overall survival. The actual efficacy of the antibody will need to be evaluated in clinical trials.

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Humanized Anti-Lewis Y Antibodies: *In Vitro* Properties and Pharmacokinetics in Rhesus Monkeys


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