Recombinant Human-Mouse Chimeric Monoclonal Antibody Specific for Common Acute Lymphocytic Leukemia Antigen

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ABSTRACT

A human-mouse chimeric antibody constructed in the present study was specific for a human tumor-associated antigen, common acute lymphocytic leukemia antigen. The antibody consisted of human heavy and light chain constant domains (γ1, and κ type) and mouse heavy and light chain variable domains, which were derived from human plasma cell leukemia line (ARH77) and mouse hybridoma cells (NL-1) specific for common acute lymphocytic leukemia antigen, respectively. The artificially fused immunoglobulin molecules were produced in mouse myeloma cells, X63Ag8.653 which were transformed with the chimeric heavy and light chain genes formed by joining the corresponding gene segments in vitro at the J-C introns. The human heavy chain enhancer element was ligated to the chimeric heavy and light chain genes, and this enhancer appeared to be obligatory for the efficient production of the chimeric antibody molecules. The stably transformed cells secreted the chimeric antibody, which specifically bound a common acute lymphocytic leukemia antigen expressing cell line. The amount of the chimeric antibody produced (10-30 μg/ml in the serum-free medium) was comparable to that made by murine hybridoma line, NL-1. The molecular weight of the chimeric heavy chain molecules was reduced from 54,000 to 50,000 upon treatment with tunicamycin, suggesting that the peptide was normally glycosylated in the transformants. The chimeric antibody exhibited complement-dependent cytotoxicity, in which glycosylation is thought to be indispensable. The antibody also mediated antibody-dependent cell-mediated cytotoxicity to the human target cells. The antibody-dependent cell-mediated cytotoxicity activity of the chimeric antibody was twice that of the murine NL-1 monoclonal antibody when human peripheral blood mononuclear cells were used as effectors.

INTRODUCTION

Monoclonal antibodies specific for tumor-associated antigens are indispensable in cancer immunotherapy and diagnosis. Human immunoglobulin is presumably superior to that of other species when administered to humans because it may function better with the recipient's effector cells and be less immunogenic. However, few human monoclonal antibodies are available because of ethical problems in in vivo immunization and difficulties in in vitro immunization required for production of human-human hybridomas. Recent advances in murine hybridoma technology have elucidated the structure and function of immunoglobulins with the exception of specific antigen recognition, which is attributable to the variable domain. If the constant domains of murine antibodies could be replaced by the human counterparts, the resultant chimeric human-mouse molecules would be expected to retain the original specificity but have a much lower antigenicity to humans. Advances in molecular biology have elucidated the structure and function of immunoglobulin genes. Technology is now available to prepare artificially fused proteins by gene manipulation. Construction of chimeric human-mouse antibodies were first reported by Boulianne et al. (3) and Morrison et al. (4) utilizing hapten specific monoclonal antibodies. They showed assembly of the chimeric antibodies, and in the former case, antigen binding activity was retained.

CALLA is a well-characterized antigen of non-T, non-B acute lymphocytic leukemia. NL-1, a mouse hybridoma line, produces antibody (γ2b, κ) specific for CALLA (5). The structure of the variable region gene segment of the heavy chain of the antibody was clarified in our laboratory and found to be highly homologous to the MOPC-21 immunoglobulin heavy chain (6, 7). We now show the structure of the NL-1 light chain gene and describe the construction of chimeric heavy and light chain immunoglobulin genes utilizing a human heavy chain enhancer. Chimeric antibody molecules were successfully made in mouse myeloma cells which bound to a CALLA-expressing cell line and killed the cells by complement-dependent cytotoxicity as well as ADCC. Our approach to preparing human-mouse chimeric antibodies to tumor-associated antigens should be advantageous in obtaining reagents suitable for clinical use.

MATERIALS AND METHODS

Cell Line. A murine hybridoma, NL-1, which secreted monoclonal antibody (γ2b, κ) specific for CALLA (5), and Manca (SK-DHL-2, human B-lymphoblastoid cell line) (5, 8), K562 (human chronic myelogenous leukemia, blast cells), and CCRF-HSB-2 (human T-acute lymphocytic leukemia) were cultured in RPMI 1640 medium containing 10% fetal calf serum. A murine myeloma cell line, X63Ag8.653 (9), was obtained from Dr. G. Köhler.

Screening of Immunoglobulin Genes. HindIII digests of NL-1 DNA were used to construct a DNA library for the variable region of the light chain gene by insertion into the Charon 28 phage vector. The gene was screened using mouse J1-3 (provided by Dr. T. Honjo, Kyoto University) as a hybridization probe. The constant region gene of the human γ light chain was screened from a library constructed by insertion of EcoRI digests of ARH77 DNA in λgtWESAB vector. Mouse C, gene (provided by Dr. T. Honjo) was used as a cross-hybridization probe. The human immunoglobulin γ1 chain was obtained as a 21-kb EcoRI fragment from a human plasma cell leukemia line, ARH77, which had

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3 The abbreviations used are: CALLA, common acute lymphocytic leukemia antigen; ADCC, antibody-dependent cell-mediated cytotoxicity; FITC, fluorescein isothiocyanate; ggt, gene encoding xanthine-guanine phosphoribosyl transferase; kb, kilobase(s); neo, gene conferring resistance to antibiotic G418; SDS, sodium dodecyl sulfate.

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been reported previously (6). The mouse heavy chain variable region gene of NL-1 hybridoma was cloned previously (7).

Transformation of Mouse Myeloma Cells. The chimeric heavy chain genes were inserted into pSV2gpt plasmid vector (10), and the chimeric light chain genes were inserted into pSV2neo vector (11). Transformation was carried out either by protoplast fusion (12) or by a DEAE-dextran method (13). Stable transformants were screened by resistance to mycophenolic acid (Lilly Co., Ltd.) for gpt and G418 (Gibco Laboratories) for neo.

Northern Blot Analysis. Total RNA was extracted from the X68Ag8.653 transformants and subjected to Northern blot analysis after electrophoresis on a denatured gel (14).

DNA Sequencing. DNA sequences were determined by a chain termination method after cloning appropriate DNA fragments on M13mpl8 and M13mpl9 phage vectors (Pharmacia) (15).

SDS-Polyacrylamide Gel Electrophoresis. The transformants were cultured for 8 h in methionine-free RPMI 1640 medium supplemented with 10% fetal calf serum. The secreted immunoglobulins were precipitated from culture fluid by goat anti-human γ antibody or goat anti-human κ antibody which bound to Protein A-Sepharose beads followed by electrophoresis on SDS-polyacrylamide gel with or without reduction by 2-mercaptoethanol.

Assay of Antigen-binding Activity. The X63Ag8.653 transformants were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Human tumor cell lines or peripheral blood lymphocytes were incubated with the culture supernatant of the transformants and then stained with FITC-labeled anti-human γ antibody (Behringerwerke AG). Binding of the chimeric antibody to the cells was examined by flow cytometry using EPICS V (Coulter).

Assay of Complement Dependent Cytotoxicity. Manca cells (1 x 10^6) were labeled with 100 μCi of 51Cr chormate (Japan Atomic Research Institute) at 37°C for 1 h in RPMI 1640 complete medium. After removing free chormate by washing, the cells were incubated with the chimeric antibody and rabbit complements for 1 h at 37°C. The cytotoxic activity was determined by 51Cr release to the supernatant. Spontaneous release was in the range of 6–13% and was subtracted from the measured values (16).

Antibody-dependent Cell-mediated Cytotoxicity. Manca cells (2 x 10^6) were labeled with 51Cr chormate and used as target cells. Human peripheral blood mononuclear cells were obtained from healthy volun-

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RECOMBINANT CHIMERIC MONOCLONAL ANTIBODY TO CALLA

Fig. 3. Construction of the chimeric immunoglobulin light chain gene. Symbols are the same as in the legend for Fig. 1. pSV2neo was used as a plasmid vector instead of pSV2gpt.

Fig. 5. SDS-polyacrylamide gel electrophoresis analysis of translated products of transformed X63Ag8.653 cells. One ml of 5 × 10⁶ cells of the transformants was cultured in the presence of 100 μCi [³⁵S]methionine for 8 h, and immunoglobulins produced were precipitated with goat anti-human γ antibody or goat anti-human κ antibody which bound to protein A-Sepharose beads (100 μl). The precipitates were applied to SDS-polyacrylamide gel electrophoresis with (a) or without (b) reduction by 2-mercaptoethanol.

Fig. 6. Northern blot analysis of the transcripts of X63Ag8.653 cells which were transformed by chimeric heavy and light chain genes. RNA was extracted and 10 μg of total RNA were electrophoresed on denatured gels and transferred to a nitrocellulose filter followed by hybridization with appropriate nick-translated probes. A, RNA blot analysis of chimeric heavy chain gene expression. The probes used were (a) mouse VH gene (PvuII-BglII) fragment and (b) human γ, heavy chain gene fragment. Lane 1 (a) NL-1 and (b) ARH77; lane 2 (a and b) X63Ag8.653; lanes 3 and 4 (a and b), transformants. B, RNA blot analysis of chimeric light chain gene expression; a mouse VNL1 gene (PstI-HindII fragment); b, human Cκ gene (Hpal-Hpal fragment); c, mouse Cκ gene used as probe. Lanes 1–4, chimeric gene transformants; lanes 5 and 6, NL1 cells; lanes 7 and 8, ARH77. One μg of polyadenylated RNA was used for ARH77.
RECOMBINANT CHIMERIC MONOCLONAL ANTIBODY TO cALLA

![Fluorescence Intensity](image)

**Fluorescence Intensity**

*Fig. 7. Binding of the chimeric antibody to Manca cells, expressing cALLA. The cells were incubated with supernatants of NL-1 or the chimeric antibody at 4°C for 30 min, washed, and stained by FITC-labeled goat anti-human γ antibody (B–E) or FITC-labeled anti-mouse γ antibody (A). Stained cells were analyzed by flow cytometry with EPICS V. A and B Manca cells; C, K562 (human chronic myelogenous leukemia, blast cells); D, CCRF-HSB2 (human T-acute lymphocytic leukemia); E, normal human peripheral blood lymphocyte. a, stained chimeric antibody or NL-1 antibody and FITC-conjugated anti-human γ or anti-mouse γ antibody, respectively; b, stained with medium and FITC-conjugated antibodies. The small positive peak found in normal peripheral blood lymphocytes was due to the presence of surface IgG* lymphocytes.*

**RESULTS**

**Construction of the Chimeric Heavy Chain Gene.** The rearranged variable region (V-D-J) gene of mouse heavy chain was cloned from NL-1 hybridoma cells which secreted monoclonal antibody specific for cALL antigen, and the constant region gene of human γ heavy chain was cloned from human plasma cell leukemia cells, ARH77. Both genes were previously reported (6, 7). The mouse variable region gene was used as an EcoRI (5’ of leader exon)-BamHI (between J2 and J3 exons) fragment for constructing a chimeric heavy chain gene (Fig. 1). The MluI-BamHI fragment of human heavy chain constant region gene was prepared from a 21-kb fragment of the cloned γ1 heavy chain gene, which also contained the human heavy chain enhancer element. The mouse variable region gene was joined to a 5’ site of the human constant region in the same transcriptional direction. The constructed chimeric gene was then inserted into vector pSV2gpt at EcoRI-BamHI sites.

**Transformation of Mouse Myeloma Cells.** X63Ag8.653, a nonproducer mouse myeloma cell line (9), was sequentially transformed by the chimeric heavy and light chain genes. The chimeric heavy chain gene was introduced into myeloma cells by a protoplast fusion method (6, 12). Protoplasts (10^9) of *Escherichia coli* MC1000 harboring the plasmid were fused with 2 x 10^6 of X63Ag8.653 cells in the presence of polyethylene glycol 4000 followed by selection with mycophenolic acid (10). The resultant stable transformants which were obtained about 3 weeks after the fusion were screened to select clones producing the gene product of the introduced chimeric heavy chain gene. Screening was done by staining the cytoplasmic human γ chain with FITC-labeled anti-human γ chain antibody. Four human γ chain producing clones were obtained. The clones producing chimeric heavy chain molecules were then used as recipient cells for transfection of the chimeric light chain gene. The chimeric light chain gene was introduced to the cells producing chimeric γ chain by a DEAE-dextran method (13). Stable transformants appearing after about 20 days as G418-resistant clones were screened for the production of the chimeric light chain gene products. Two positive clones...
producing chimeric heavy and light chains were established from 2 x 10^6 cells. Products of the recloned transformants were used for further analyses.

Analysis of Transcripts. Transcripts of introduced genes in the stable transformants were analyzed by Northern blot analysis. As shown in Fig. 4A, a band of 1.8 kb in length appeared with RNAs of chimeric gene-transformed cells when the human C\textsubscript{\gamma} probe (PstI-PstI fragment) was used. The probe gave no such band in RNAs extracted from X63Ag8.653. The variable exon probe of the mouse heavy chain gene (PvuII-BglII fragment) gave the same hybridizing band as the C\textsubscript{\gamma} probe. Hence, the 1.8-kb RNA was assigned to the secretory type mRNA of the chimeric heavy chain gene. Amounts of mRNAs in chimeric gene-transformed myeloma cells were 3–4 times higher than those in hybridomas. Similar observations were obtained for \kappa chain gene transcripts (Fig. 4B). The human \kappa constant region probe (HpaI-HpaI) gave a band of about 1.3 kb in length. The band of the same size was observed when the blotted filter was hybridized with the variable exon probe (PstI-HincII) of the mouse \kappa chain gene. Northern blot analysis revealed that the introduced chimeric heavy and light chain genes were transcribed and processed accurately in X63Ag8.653 and might yield functional mRNAs of the chimeric immunoglobulin genes.

Analysis of Translation Products. Immunoglobulin production was examined in the culture supernatants of X63Ag8.653 transformed by the chimeric genes. The stable transformants were cultured in [35S]methionine-containing medium for 8 h. An affinity-purified goat antibody specific for human \gamma chain was bound to Protein A-Sepharose containing medium and added to the culture medium. The precipitates were applied to SDS-polyacrylamide gel electrophoresis giving a band corresponding to the assembled protein of H\textsubscript{\gamma}L\textsubscript{\delta} at nonreducing conditions (Fig. 5b). Two bands appeared on SDS-polyacrylamide gel electrophoresis at the place corresponding to \gamma heavy and light chains when the sample was applied after reduction by 2-mercaptoethanol (Fig. 5a). These results indicated that the introduced chimeric genes were transcribed, processed, and translated to yield the chimeric immunoglobulin light and heavy chain peptide, which associated together to form the natural tetrameric molecules H\textsubscript{\gamma}L\textsubscript{\delta} of IgG.

Glycosylation of Immunoglobulin \gamma Peptide. Human immunoglobulin \gamma\textsubscript{\textit{i}} peptides possess sites of \textit{in vivo} glycosylation that are sensitive to tunicamycin treatment. The X63Ag8.653 transformants were cultured in RPMI 1640 complete medium supplemented with 1 \mu g/ml tunicamycin and [35S]methionine for 16 h at 37°C, and the resulting immunoglobulin molecules were analyzed by SDS-polyacrylamide gel electrophoresis after reduction with 2-mercaptoethanol (Fig. 6). The band of M, 50,000 in size appeared in addition to a faint band of M, 54,000 corresponding to the \gamma\textsubscript{\textit{i}} chain. The transformants cultured without addition of tunicamycin gave only the M, 54,000 band. In the case of ARH77 cells, the human \gamma\textsubscript{i} gene was cloned, the M, 54,000 band shifted to M, 54,000 upon addition of tunicamycin to the culture medium (data not shown). These data suggested that the products of the chimeric genes were glycosylated similarly to native human \gamma chains.

Binding Activity of the Chimeric Antibody to CALLA Antigen. X63Ag8.653 transformants secreted the assembled antibodies to the culture fluid. The binding activity of the antibodies to the CALL antigen was assayed using Manca cells as targets. This is a human B-lymphoblastoid cell line (8) which expresses IgM (\kappa, \kappa) and CALLA antigen. The culture supernatant of the transformed cells was incubated with Manca cells followed by staining with FITC-labeled goat antibody specific for human \gamma heavy chain. The staining was then assessed by flow cytometry (Fig. 7b). The chimeric antibody in the culture supernatants of X63Ag8.653 transformants bound to Manca cells. The binding activity was comparable to the mouse monoclonal antibody.
RECOMBINANT CHIMERIC MONOCLONAL ANTIBODY TO cALLA

from NL-1 hybridoma (Fig. 7A). The chimeric antibody did not react with human tumor cell lines, K562 (chronic myelogenous leukemia, blast cells), and CCRF-HSB2 (T-acute lymphocytic leukemia) or with normal human peripheral blood lymphocytes (Fig. 7C-E), which were lacking cALLA. No positive binding was observed in the culture supernatants of X63Ag8.653 cells or X63Ag8.653 cells transformed with the chimeric heavy chain gene alone.

Complement-dependent Cytotoxicity. Human IgG1 subclass antibodies mediate complement-dependent cytotoxicity. The culture fluids of X63Ag8.653 transformants were added to the labeled Manca cells together with rabbit complement (Fig. 8). The complement used in this experiment caused 11% 51Cr release, and the antibody alone exhibited infrequent lytic activity (0.9%) in the absence of complement. The antibody solution of the chimeric gene-transformed cells were clearly cytotoxic to Manca cells in the presence of complement. The monoclonal antibody obtained from murine NL-1 hybridoma showed twice this level of cytotoxicity. The chimeric or NL-1 antibody was not cytotoxic to K562 or CCRF-HSB2 (data not shown).

Antibody-dependent Cell-mediated Cytotoxicity by the Chimeric Antibody. ADCC is an important function of certain classes of antibodies, including IgG. The chimeric antibody possessing the constant region of human origin was tested for ADCC activity using human effector cells. Normal human peripheral blood mononuclear cells were used as effector cells (Fig. 9). The antibody of the chimeric gene transformants, which showed low but significant activity in the complement-dependent cytotoxic assay, exhibited twice the ADCC activity as the same concentration of monoclonal antibody of the murine NL-1 hybridoma. These results suggest that our chimeric antibodies may function as effector molecules in antibody-dependent cell-mediated cytotoxicity in vivo.

DISCUSSION

We have made a human-mouse chimeric antibody consisting of murine variable region and human constant region domains. The chimeric immunoglobulins were produced by transformed mammalian cells. Chimeric immunoglobulin genes of heavy and light chains were sequentially introduced to a mouse myeloma. The introduced genes were transcribed and spliced accurately, and the resultant mRNAs were translated to immunoglobulin heavy and light chain peptides. These were assembled together to yield a natural form of IgG and secreted into the culture fluid. The chimeric antibody retained its specificity for cALLA, which was defined by the variable region genes (VH and VL) of mouse NL-1 hybridoma, and the antibody molecules with human γ and κ constant domains demonstrated binding to cells expressing cALLA. The carbohydrate moiety of immunoglobulin molecules is reported to be indispensable for some physiological functions, i.e., activation of complements and ADCC (17). From this point of view, antibodies produced by mammalian cells should be superior to those produced by complementary DNA-type gene transfected to bacterial cells. The molecular weight of the chimeric heavy chain changed from 54,000 to 50,000 upon treatment of the transformed cells with tunicamycin, indicating that glycosylation occurred normally. The chimeric antibody caused the release of 51Cr from labeled cells in the presence of complement, indicating that the constant region functioned in complement-dependent cytotoxicity. ADCC is an important physiological function attributed to certain classes of antibodies and has been implicated in self defense against malignant cells. Thus, retention of this activity is strongly desirable for antibodies intended for clinical treatment. When the same amounts of antibodies were used, the NL-1 monoclonal antibody (mouse γ2a, κ) showed higher activity in complement-dependent cytotoxicity than did the chimeric antibody, whereas the same concentration of chimeric antibody exhibited strikingly higher ADCC activity. This may be due to the effectiveness of interaction between the human constant domains and human effector cells. High ADCC activity may be particularly beneficial for immunotherapy of cancer patients.

Boulianne et al. (3) reported a human-mouse chimeric antibody (μ, κ) specific for haptenic antigen, trinitrophenyl. They observed that the transformed cells produced pentameric molecules, which exhibited similar specificity to trinitrophenyl with nearly equal affinity to that of the mouse hybridoma from which the variable region genes were isolated. Morrison et al. (4) reported human-mouse chimeric antibody of γγ type. The variable region genes were obtained from SI07, specific for phosphorylcholine hapten. J558L and P3 mouse myeloma cells were transformed by the chimeric genes and assembled immunoglobulins of tetrameric form (H2L2) were produced. The present study demonstrated the preparation of a human-mouse chimeric antibody exhibiting specificity to human tumor-associated antigen of potential clinical importance. The X63Ag8.653 transformants yielded satisfactory amounts of chimeric antibody (10–30 μg/ml in the serum-free medium) and more concentrated chimeric antibodies were obtained in murine ascites. This high expression of chimeric heavy and light chain genes in murine myeloma cells may be due to the presence of a human heavy chain enhancer element in the gene constructs (6). Very recently, a human-mouse chimeric antibody against a human breast cancer antigen has been reported (18). Assembly of any other variable region genes to human Cγ1 and Cκ genes with the human enhancer is possible and such human chimeric antibodies offer a promising replacement for conventional murine monoclonal antibodies.

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