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Recombinant Human-Mouse Chimeric Monoclonal Antibody Specific for Common Acute Lymphocytic Leukemia Antigen[1]

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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 (γ_1 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 resulted in many promising reagents and some are already being used in clinical trials. Since multiple injections of the antibodies are necessary in immunotherapy protocols, reduction in antigenicity is an important concern. Administration of heterologous monoclonal antibodies may lead to allergic reactions and neutralization of the administrated antibodies.

Immunoglobulin molecules consist of variable and constant region domains. The constant domains of mouse immunoglobulin molecules are probably an important target of antigenic recognition by the human immune system (1). This portion of the molecule is responsible for many physiological functions 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 (2). 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[3] is a well-characterized antigen of non-T, non-B acute lymphocytic leukemia. NL-1, a mouse hybridoma line, produces antibody (γ_{2a}, 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 (γ_{2a}, 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. *Hind*III digests of NL-1 DNA were used to contruct 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 $J_{\kappa 1-5}$ (provided by Dr. T. Honjo, Kyoto University) as a hybridization probe. The constant region gene of the human κ light chain was screened from a library constructed by insertion of *Eco*RI digests of ARH77 DNA in λgtWESλB vector. Mouse C_κ gene (provided by Dr. T. Honjo) was used as a cross-hybridization probe. The human immunoglobulin γ_1 chain was obtained as a 21-kb *Eco*RI 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; *gpt*, 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 pSV2*gpt* plasmid vector (10), and the chimeric light chain genes were inserted into pSV2*neo* 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 M13mp18 and M13mp19 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 in the presence of [^{35}S]methionine (Amersham). 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 and 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 (1×10^7)

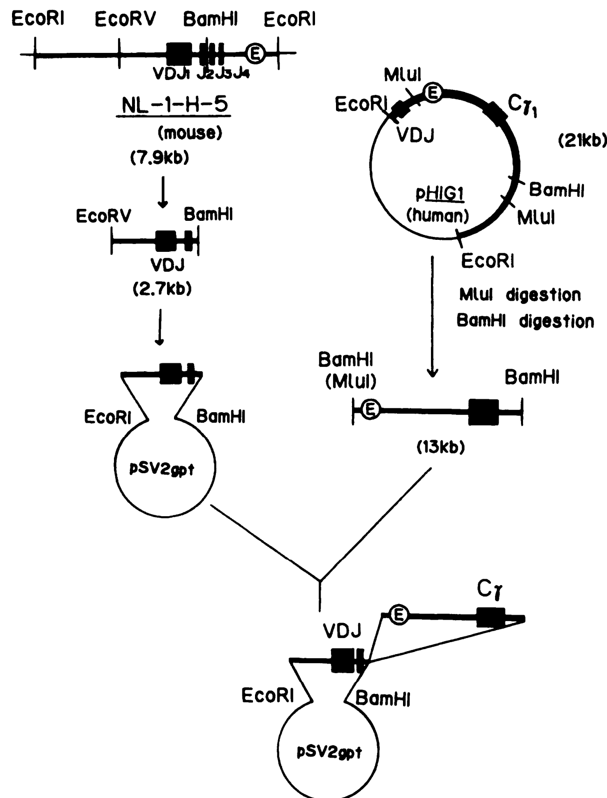
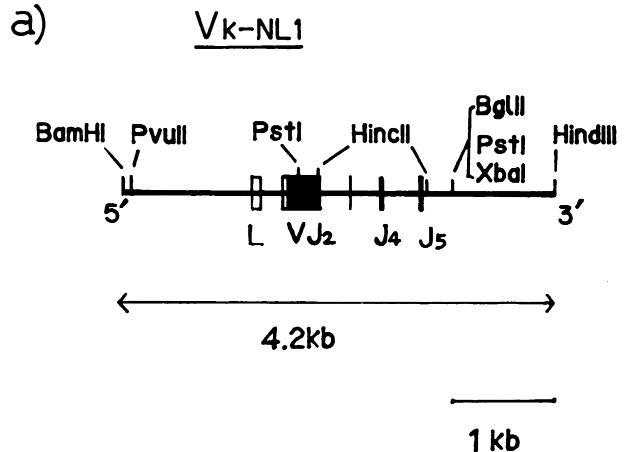


Fig. 1. Construction of the chimeric immunoglobulin heavy chain gene. *Boxes*, exon regions of human and mouse immunoglobulin heavy chain genes (6, 7); *thick lines*, intron DNA; *thin lines*, vector DNA. Ⓔ, position of the human heavy chain enhancer element. pSV2gpt was used as a vector for transforming mouse cells.



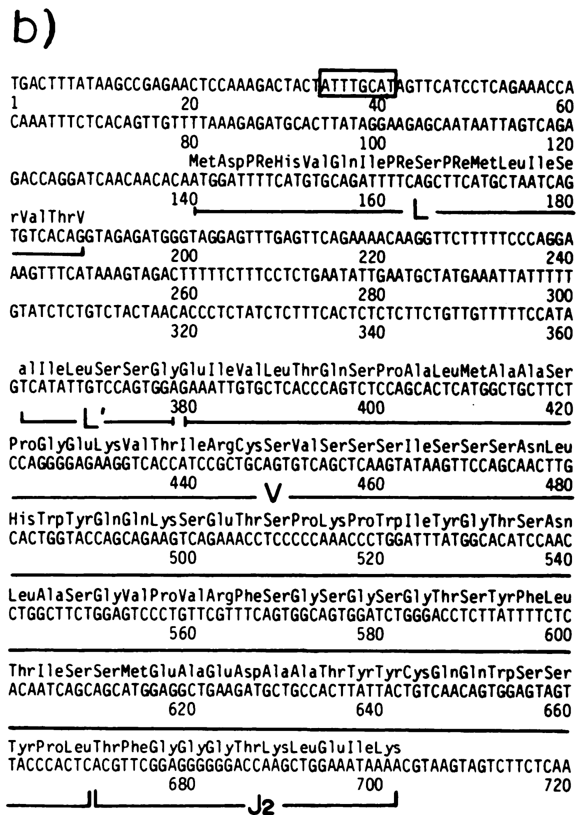


Fig. 2. Structure and nucleotide sequences of mouse κ chain variable region gene (V_κ) derived from mouse hybridoma, NL-1, specific for cALLA. *a*, restriction enzyme map of $V_{\kappa NL\text{-}1}$ gene; *b*, nucleotide sequences and deduced amino acid sequences of the variable region of NL-1 κ chain gene. The consensus octamer sequence of immunoglobulin gene is *boxed*.

were labeled with 100 μCi of ^{51}Cr chromate (Japan Atomic Research Institute) at 37°C for 1 h in RPMI 1640 complete medium. After removing free chromate 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 ^{51}Cr 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 (2×10^6) were labeled with ^{51}Cr chromate and used as target cells. Human peripheral blood mononuclear cells were obtained from healthy volun-

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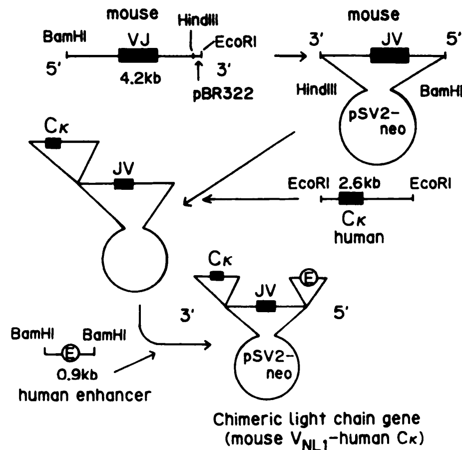


Fig. 3. Construction of the chimeric immunoglobulin light chain gene. *Symbols* are the same as in the legend for Fig. 1. pSV2*neo* was used as a plasmid vector instead of pSV2*gpt*.

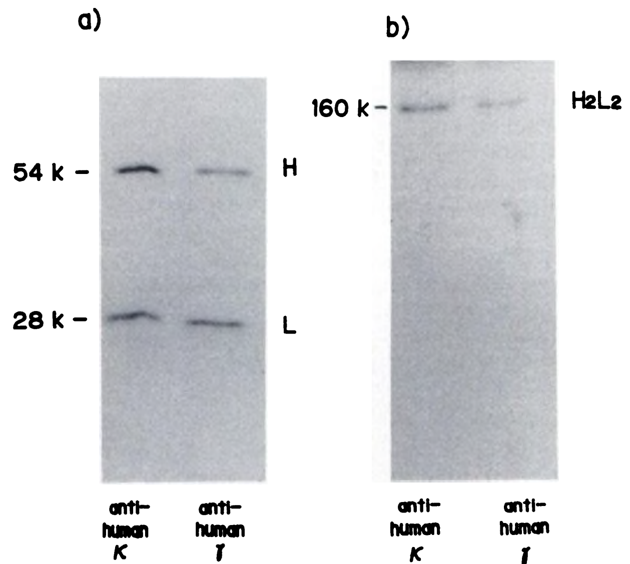
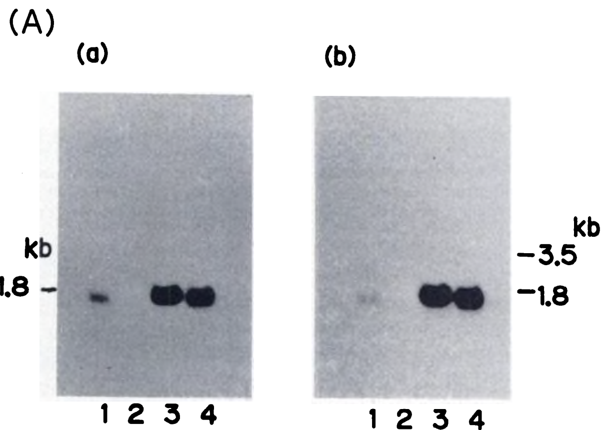


Fig. 5. SDS-polyacrylamide gel electrophoresis analysis of translated products of transformed X63Ag8.653 cells. One ml of 5 × 10^6 cells of the transformants was cultured in the presence of 100 μCi [^{35}S]methionine for 8 h, and immunoglobulins produced were precipitated with goat anti-human γ antibody 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.



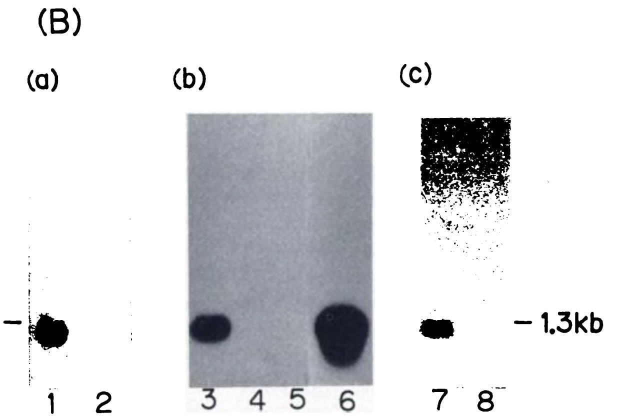


Fig. 4. Northern blot analysis of the transcripts of X63Ag8.653 cells which were transformed by chimeric heavy and light chain genes. RNA was extracted and 10 μg of total RNA were electrophoresed on denatured gels and transferred to a nitrocellulose filter followed by hybridization with appropriate nick-translated probes. *A*, RNA blot analysis of chimeric heavy chain gene expression. The probes used were (*a*) mouse V_H gene (*Pvu*II-*Bgl*II) fragment and (*b*) human γ_1 heavy chain gene (*Pst*I-*Pst*I 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 $V_{\kappa NL\text{-}1}$ gene (*Pst*I-*Hinc*II fragment); *b*, human C_κ gene (*Hpa*I-*Hpa*I fragment); *c*, mouse C_κ gene used as probes. *Lanes 1*, *3*, and *8*, chimeric gene transformants; *lanes 2* and *4*, X63Ag8.653; *lanes 5* and *7*, NL1 cells; *lane 6*, ARH77. One μg of polyadenylated RNA was used for ARH77.

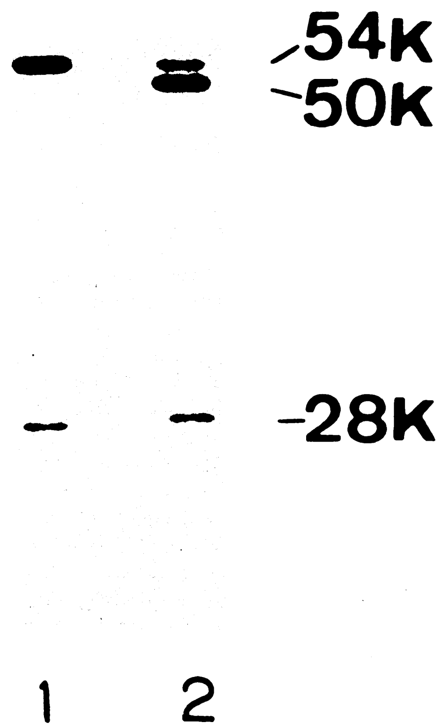


Fig. 6. Effects of tunicamycin treatment of the X63Ag8.653 transformants on the produced immunoglobulin. The transformants were cultured in the presence (*lane 2*) or absence (*lane 1*) of tunicamycin for 16 h in the medium supplemented with [^{35}S]methionine. Immunoglobulins were precipitated with goat anti-human γ antibody as described in Fig. 5. *K*, thousands.

teers and separated by using lymphocyte separation medium (Bionetics Laboratory Products). Target cells (2 × 10^4; 50 μl) were mixed with 100 μl of effector cells at ratios of 1:50, 1:20, and 1:5 together with 50 μl of the chimeric or NL-1 antibody solution (1 μg/ml). Incubation was

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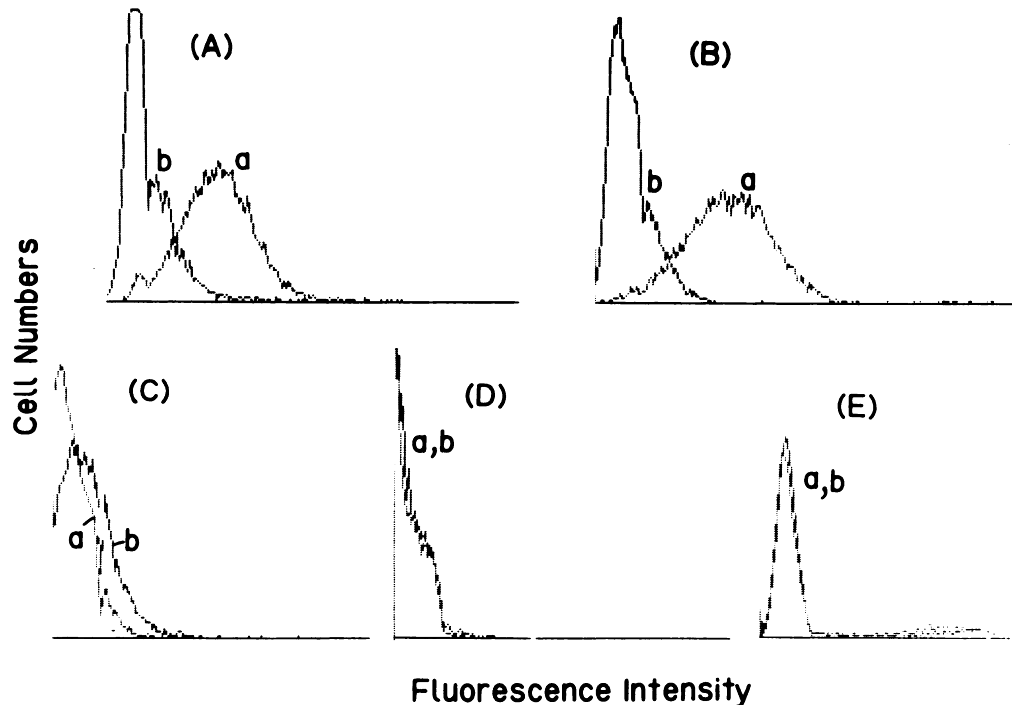

Fig. 7. Binding of the chimeric antibody to Manca cells, expressing cALLA. The cells were incubated with supernatants of NL-1 or the chimeric antibody at 4°C for 30 min, washed, and stained by FITC-labeled goat anti-human γ antibody (*B–E*) or FITC-labeled anti-mouse γ antibody (*A*). Stained cells were 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.

done at 37°C for 6 h under a 5% CO_2 atmosphere. Released ^{51}Cr was measured to determine the cytotoxic activity (17).

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_1 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 *Eco*RI (5′ of leader exon)-*Bam*HI (between J_2 and J_3 exons) fragment for constructing a chimeric heavy chain gene (Fig. 1). The *Mlu*I-*Bam*HI fragment of human heavy chain constant region gene was prepared from a 21-kb fragment of the cloned γ_1 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 pSV2*gpt* at *Eco*RI-*Bam*HI sites.

Construction of the Chimeric Light Chain Gene. The light chain variable region gene was cloned from genomic DNA of mouse hybridoma, NL-1, as a 6.5-kb *Hind*III fragment using the Charon 28 library. The DNA base sequence of the cloned gene was determined (Fig. 2). The variable region gene had rearranged to the J_2 segment. The constant region gene of the human κ light chain gene was a 2.6-kb *Eco*RI fragment which was cloned from the λgtWESλB genomic library of ARH77 using mouse constant region gene as a cross-hybridization probe. The mouse variable region gene was trimmed to a *Bam*HI-*Hind*III fragment and a small *Hind*III-*Eco*RI fragment of pBR322 was added to the 3′ site of the variable region gene segment. The resultant *Eco*RI-*Bam*HI fragment of mouse variable region gene was cloned into pSV2*neo* (Fig. 3). The human κ chain constant region gene was then inserted into an *Eco*RI site of pSV2*neo* vector containing mouse variable region gene. The human heavy chain enhancer segment (*Mlu*I-*Hpa*I) was ligated to the *Bam*HI site (5′ end of the variable region gene) of the recombinant plasmid with the aid of an oligonucleotide synthetic linker.

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 2×10^6 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

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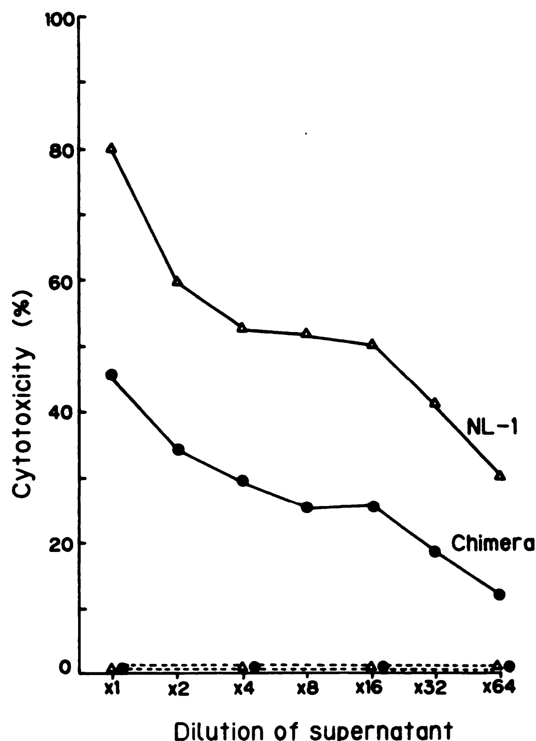

Fig. 8. Complement-dependent cytotoxicity. Manca cells (100 μl; 4×10^5) labeled with ^{51}Cr chromate, were incubated for 30 min at 37°C with 100 μl of chimeric antibody solution (1 μg/ml) of the X63Ag8.653 transformants (●) or NL-1 monoclonal antibody solution (1 μg/ml) (Δ) in the presence (——) or absence (----) of rabbit complement. ^{51}Cr release from the Manca cells was measured and the percentage of cytotoxic activity was calculated after subtracting spontaneous ^{51}Cr release. Rabbit complement alone caused 11% ^{51}Cr release.

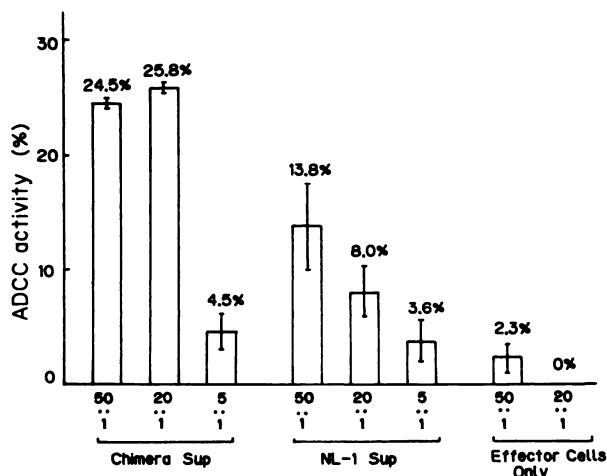

Fig. 9. ADCC. Manca cells (2×10^4 in 50 μl) labeled with ^{51}Cr chromate were incubated for 6 h with 50 μl of the chimeric antibody or NL-1 antibody and 100 μl of the human effector cells at effector:tumor cell ratios as indicated. Antibody concentration used was 1 μg/ml. Amounts of ^{51}Cr released were measured in triplicate assays, and the percentage of cytotoxicity was calculated after subtraction of spontaneous release (6–13%). The chimeric antibody did not show any ADCC activity against CCRF-HSB2 cells which were cALLA negative. *Sup*, supernatant containing antibody (1 μg/ml); *bars*, SD.

producing chimeric heavy and light chains were established from 2×10^6 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. 4*A*, a band of 1.8 kb in length appeared with RNAs of chimeric gene-transformed cells when the human C_{γ_1} probe (*Pst*I-*Pst*I 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 (*Pvu*II-*Bgl*II fragment) gave the same hybridizing band as the C_{γ_1} 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 κ chain gene transcripts (Fig. 4*B*). The human κ constant region probe (*Hpa*I-*Hpa*I) 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 (*Pst*I-*Hinc*II) of the mouse κ 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 [^{35}S]methionine-containing medium for 8 h. An affinity-purified goat antibody specific for human γ chain was bound to Protein A-Sepharose beads and added to the cultured medium. The precipitates were applied to SDS-polyacrylamide gel electrophoresis giving a band corresponding to the assembled protein of H_2L_2 at nonreducing conditions (Fig. 5*b*). Two bands appeared on SDS-polyacrylamide gel electrophoresis at the place corresponding to γ heavy and light chains when the sample was applied after reduction by 2-mercaptoethanol (Fig. 5*a*). 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_2L_2 of IgG.

Glycosylation of Immunoglobulin γ Peptide. Human immunoglobulin γ_1 peptides possess sites of *in vivo* glycosylation that are sensitive to tunicamycin treatment. The X63Ag8.653 transformants were cultured in RPMI 1640 complete medium supplemented with 1 μg/ml tunicamycin and [^{35}S]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_r 50,000 in size appeared in addition to a faint band of M_r 54,000 corresponding to the γ_1 chain. The transformants cultured without addition of tunicamycin gave only the M_r 54,000 band. In the case of ARH77 cells, where the human γ_1 gene was cloned, the M_r 54,000 band shifted to M_r 50,000 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 γ chains.

Binding Activity of the Chimeric Antibody to cALL 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 (μ, κ) and cALL antigen. The culture supernatant of the transformed cells was incubated with Manca cells followed by staining with FITC-labeled goat antibody specific for human γ heavy chain. The staining was then assessed by flow cytometry (Fig. 7*B*). 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

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