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# CONSTRUCTION, EXPRESSION, AND BIOLOGIC ACTIVITY OF MURINE/HUMAN CHIMERIC ANTIBODIES WITH SPECIFICITY FOR THE HUMAN $\alpha/\beta$ T CELL RECEPTOR

CLYDE W. SHEARMAN,[1*] E. J. KANZY,[†] DAWSON K. LAWRIE,[*] YI-WAN LI,[2*] PALLAIAH THAMMANA,[3*] GORDON P. MOORE,[4*] AND ROLAND KURRLE[†]

*From *Genzyme Corporation, Framingham, MA 01701; and †Behringwerke Aktiengesellschaft, D-3550 Marburg, Federal Republic of Germany*

**Murine/human chimeric antibodies with specificity for the human TCR-$\alpha/\beta$ have been produced by genetic engineering. The L and H chain V region exons encoding the murine mAb BMA 031 were isolated and inserted into mammalian expression vectors containing the human $\kappa$ and $\gamma$1 or $\gamma$4 C region exons. The chimeric genes were transfected into murine Sp2/0 hybridoma cells by electroporation and transfectomas secreting chimeric antibody were isolated. Secretion levels ranged from 1 to 7 pg/cell/24 h. The chimeric antibodies bound specifically to T cells and competed effectively with the parental murine mAb for binding to these sites. The ability to promote antibody-dependent cell-mediated cytolysis was significantly enhanced in the chimeric antibodies as compared with murine BMA 031. C-dependent cytolysis, however, was not detectable with any of the antibodies. Chimeric BMA 031 is a clinically relevant, genetically engineered antibody with potential uses in transplantation, graft-vs-host disease, autoimmune diseases and other T cell-related disorders.**

mAb directed against human T cells have been used extensively to analyze mechanisms of T cell activation and human T cell imbalances in various diseases (1–3). Furthermore, some of these antibodies seem to be powerful therapeutic agents in the treatment of leukemias, renal allograft rejection, acute graft-vs-host disease and autoimmune diseases (4–6).

Despite the fact that mAb directed against T cells have been applied to patients for almost 10 yr (7, 8), their in vivo mechanisms of action are not yet completely understood. The particular Ag recognized by the mAb seems to play a crucial role in its therapeutic effectiveness. Also, in addition to the specificity of the mAb, Fc-mediated reactions influence in vitro as well as in vivo effects. Therefore, the selection of an appropriate antibody isotype may be as important for therapeutic efficacy as the selection of an appropriate specificity.

The human TCR-$\alpha/\beta$ is expressed on all mature T lymphocytes and is thought to recognize Ag in the context of MHC molecules. This heterodimer is noncovalently linked to the multi-chain CD3-Ag complex, which is strongly involved in signal transduction and amplification after binding of Ag to the TCR-$\alpha/\beta$ (9). Under in vitro experimental conditions, T cells can be activated by providing trigger signals via the CD3-Ag complex. Binding of anti-CD3 mAb, like OKT3 or BMA 030, followed by physiologic or artificial cross-linking of the mAb usually results in polyclonal T cell activation (4). Under physiologic conditions, binding of mAb to the CD3-Ag complex results in a so-called "signal one" of T cell activation. A second activation signal is derived from accessory cells, provided the Fc part of the Ig molecule is able to interact with FcR on the accessory cells (10). Only under conditions in which both signals are available is T cell activation complete, resulting in lymphocyte proliferation (11, 12).

Until recently, all mAb directed to epitopes of the CD3/TCR complex were thought to deliver the first signal of T cell activation. However, in vitro studies with the anti-human TCR-$\alpha/\beta$ mAb BMA 031 have shown that this antibody delivers different signals of T cell activation than anti-CD3 mAb even of the same isotype (13). These differences are most likely caused by the specificities of these mAb. In addition, because BMA 031 is of the murine IgG2b isotype and thus does not interact with the majority of human FcR (10), binding of BMA 031 to human TCR usually does not result in induction of secondary signals from accessory cells. This leads to incomplete T cell activation, weak T cell proliferation, and the release of very small amounts of cytokines. These properties seem to be essential with respect to clinical application. In clinical trials, where BMA 031 is used for prophylaxis and therapy of both graft rejections in organ transplantation and graft-vs-host disease after bone marrow transplantation, this antibody is very well tolerated (14, 15).

Most mAb used in human therapy are of murine origin and may have limited utility because they usually elicit an immune response in patients (16, 17). Such a response reduces therapeutic efficacy and also may cause undesired clinical side effects. Human mAb are expected to circumvent this problem but they are difficult to produce

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[1] Address correspondence and reprint requests to C. W. Shearman, Genzyme Corporation, One Mountain Road, Framingham, MA 01701.
[2] Present address: Chinese Academy of Medical Sciences, Beijing, China.
[3] Present address: E.I. DuPont de Nemours and Co., Glasgow Site, Building 700, Newark, DE 19717.
[4] Present address: Department of Molecular Genetics, Smith Kline Beecham, 709 Swedeland Road, King of Prussia, PA 19406.



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and often have low affinity. Moreover, human hybridomas are usually unstable and secrete Ig at low levels (18, 19). Chimeric antibodies consisting of murine V regions and human C regions represent a compromise. Conceptually, a chimeric antibody would retain the affinity and specificity of the parental murine mAb, and would eliminate the patient immune response to the murine C regions. Also, transfectomas producing chimeric antibodies appear to be stable and secrete antibody at levels sufficient for commercial application (20, 21). A number of chimeric antibodies have been produced recently (22–24) and some are entering clinical trials (25). Here, we report the production of chimeric antibodies with the affinity and specificity of BMA 031, a murine mAb specific to the human TCR-$\alpha/\beta$ (4). These antibodies were used to study the Fc-mediated components of T cell activation. Moreover, chimeric BMA 031 antibodies also display enhanced ADCC[5] activity and may have advantages in vivo, especially in situations in which elimination of T cells is required. Chimeric BMA 031 may, therefore, have efficacy for both immunoregulation and treatment of T cell-related disorders, like T cell leukemias.

## MATERIALS AND METHODS

*Cell culture.* The BMA 031, P3x63.Ag8, and Sp2/0-Ag14 hybridomas were cultured in DMEM supplemented with 10% FCS, 10 mM HEPES (pH, 7.3), 2 mM L-glutamine, 10 mM nonessential amino acids (GIBCO, Grand Island, NY), and 10 mM pyruvate. Transfectomas were grown in the above medium containing 1 μg/ml mycophenolic acid, 50 μg/ml xanthine, and 500 μg/ml Geneticin (GIBCO). For antibody production, hybridoma or transfectoma cells were cultured in serum-free Iscove's medium. HPB-ALL and CEM cells were also cultured in serum-free Iscove's medium. All lines were maintained at 37°C in 5 to 7% $CO_2$.

*Nucleotide sequencing.* The nucleotide sequence of both the H and L chain V regions of BMA 031 mRNA was determined by the primer extension method (27) using avian myeloblastosis virus reverse transcriptase. Total RNA was extracted from BMA 031 cells with guanidinium thiocyanate (28) and poly $A^+$ mRNA was isolated by oligo (dT) cellulose chromatography (29). Universal primers, corresponding to the C regions of H and κL chains, were used in the initial sequencing (30). DNA sequencing of the cloned VH and VL regions was performed directly on pUC subclones by using universal forward and reverse primers (31). Additional primers were synthesized on an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer to complete the sequencing.

*Isolation of BMA 031 $V_H$ and $V_L$ regions from a genomic library.* A genomic DNA library from the BMA 031 cell line was constructed in the λ phage vector EMBL-3. High m.w. DNA was isolated, partially digested with restriction endonuclease Sau3A, and fractionated by agarose gel electrophoresis (32). DNA fragments between 9 and 23 kb were eluted onto a glass fiber filter, extracted, and ethanol precipitated. The DNA was then ligated with λ EMBL-3 that had been digested with *Bam*HI and dephosphorylated. Recombinant λ phage were packaged with GIGAPACK GOLD extract (Stratagene, San Diego, CA) and plated at a density of 4.4 × $10^5$/150 mm diameter petri dish. Duplicate filter lifts were prepared by using nitrocellulose filters (Schleicher and Schuell, Keene, NH). Filters were hybridized with random oligodeoxynucleotide-labeled probes (33). The probe used for identifying the $V_H$ region was the 1.3 kb *Hin*dIII/*Pst*I DNA fragment (HPH) containing murine $J_{H4}$ and a portion of the intron between the V and C region of the H chain. The probe used for detecting the $V_L$ region was the 1.1-kb *Pst*I/*Hin*dIII DNA fragment (HPL) derived from the murine L chain intron (see Fig. 1). Putative positive clones were isolated and purified by up to four rounds of rescreening.

*Transfection of DNA into mouse cells by electroporation.* DNA was introduced into murine hybridoma Sp2/0-Ag14 cells by electroporation. 1 to 2 × $10^7$ actively growing Sp2/0-Ag14 cells were washed and resuspended in 1.0 ml of sterile PBS. Fifteen micrograms of each chimeric, Igκ and IgG1 (or IgG4), plasmid (linearized with *Bam*HI) were added to the cell suspension. The DNA/cells were transferred to a precooled shocking cuvette, incubated on ice at least 5 min and then a 0.5 kv/cm electric pulse was delivered for 10 ms (Transfector 300, BTX, San Diego, CA). After shocking, the DNA/cell mixture was returned to ice for 10 min and then diluted in 40 ml of supplemented DMEM and incubated at room temperature for 10 min. Finally, the cells were transferred to a 37°C incubator with 7% $CO_2$ for 48 h before plating in selective medium, containing 1 μg/ml mycophenolic acid, 50 μg/ml xanthine and 1 mg/ml Geneticin. Cells were plated in 96-well plates at 3 × $10^4$ cells/well.

*T cell proliferation assay.* Ficoll-separated PBL were cultured in serum-free Iscove's medium at 5 × $10^4$ cells/well in 96-well U-shape microtiter plates in the presence of various concentrations of anti-CD3 or BMA 031 antibodies (0 to 10 μg/ml). Cells were cultured in quadruplicate for 3 or 6 days at 37°C. [$^{14}$C]TdR (75 nCi/well; New England Nuclear, Boston, MA) was added during the last 16 h of culture. Cells were collected by an automated cell harvester and analyzed by an automatic filter counting system (Innotech Trace 96, Innotech, Trumbull, CT).

*Cytofluorometric assays for specificity and affinity.* Heparinized HPB cells from healthy volunteers were incubated at 4°C for 30 min in the presence of BMA 031 culture supernatants, purified Ig (BMA 031, BMA 031-G1, BMA 031-G4), or with FITC-conjugated BMA 031 antibodies (direct immunofluorescence assay). In indirect assays, FITC-conjugated rabbit anti-mouse Ig F(ab')$_2$ or anti-human Ig F(ab')$_2$ antibodies were used as second-step reagents. In several experiments, cells were preincubated with polyclonal human Ig (Beriglobin, Behringwerke, Germany) to reduce nonspecific binding of mAb to FcR. All mAb were used at concentrations twice that required for Ag saturation. To analyze the relative affinities of murine and chimeric BMA 031 antibodies, competitive immunofluorescence assays were carried out. PBMC were separated by Ficoll-Hypaque density gradient centrifugation and incubated with mAb at various concentrations (0.01 to 10 μg/ml) for 30 min. After removing unbound antibodies by two washing steps, cells were incubated with 10 μg/ml of FITC-conjugated BMA 031 antibodies for 30 min. Cells from all experiments were analyzed either on an Ortho (Raritan, NJ) Cytofluorograph 50H/2150 computer system or on a Becton Dickinson (Mountain View, CA) FACStar Plus as described elsewhere (26). The percentage of fluorescein-positive cells was calculated by modified Ortho or standard FACStar Plus software.

*Cytotoxicity assays.* To measure the cytolytic capacity of the BMA 031 antibody preparations, a 20-h $^{51}$Cr-release assay was performed to measure ADCC and NK activity. $^{51}$Cr-labeled HPB-ALL target cells were incubated with (ADCC) or without (NK activity) various concentrations of antibodies for 20 h in the presence of Ficoll-separated PBL (effector cells). TCR-$\alpha/\beta$-negative CEM cells were used as control target cells. The antibodies were allowed to bind first to target cells (30 min) before the effector cells were added. The E:T ratio varied from 1:1 to 50:1. Cytolysis in the absence of antibodies was considered to be caused by NK activity. The percentage of specific lysis was calculated as described earlier (34). Spontaneous $^{51}$Cr release in the absence of effector cells was always less than 5%. All samples were analyzed in triplicate. CDC was measured in a standard lymphocytotoxicity test as used routinely to measure AHLG-cytolysis titers (Pressimmune, Behringwerke, Germany) (35).

*Preparation of F(ab')$_2$ molecules.* BMA 031-G1 and BMA 031-G4 (1 mg/ml) were cleaved with pepsin (9000 FIP-U/g, Merck, Darmstadt, Germany) at an antibody to pepsin ratio of 20:1. BMA 031-G1 was digested in 100 mM sodium citrate (pH 3.5) at 37°C for 30 min. BMA 031-G4 was digested in 100 mM sodium citrate (pH 4.0) at 37°C for 2 h. Digestion was stopped by adjusting the pH to 7.5 with 2 M Tris-HCl. After concentration with a Centricon 30 (Amicon, Danvers, MA) IgG F(ab')$_2$ was separated from intact IgG and smaller fragments by size exclusion chromatography on Ultragel AcA34 (IBF Biotechnics, Savage, MD) by using 20 mM Tris-HCl (pH 7.5) and 400 mM NaCl. The F(ab')$_2$ fraction was pooled and passed over a protein A Sepharose CL4B (Pharmacia, Piscataway, NJ) column in 50 mM Tris-HCl (pH 8.6) and 150 mM NaCl. Unbound protein was concentrated by ultrafiltration and dialyzed against 10 mM sodium phosphate (pH 7.2) and 140 mM NaCl. Purity of the IgG F(ab')$_2$ preparations was checked by SDS-PAGE and an ELISA by using mAb specific for the Fc portion of human IgG1 or IgG4.

## RESULTS

*Restriction analysis and mRNA sequencing of the BMA 031 V regions.* Genomic DNA from BMA 031 and its fusion partner, P3x63.Ag8, was digested with various restriction endonucleases and analyzed by Southern blotting to identify the functionally rearranged V regions.

[5] Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytolysis; ALL, acute lymphocytic leukemia; CDC, C-dependent cytolysis; CDR, complementarity-determining region; HPB, human peripheral blood; $V_H$, variable H chain; $V_L$, variable L chain.

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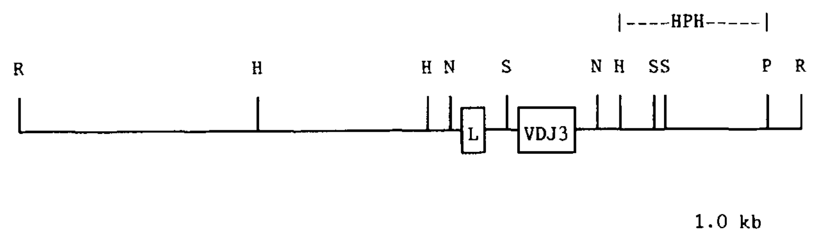
This analysis showed that the H chain V region was contained in a 5.6-kb *Eco*RI fragment and that the L chain V region was in a 3.0-kb *Hin*dIII fragment. Partial restriction enzyme maps (later confirmed and extended by DNA sequencing of the genomic clones) are shown in Figure 1. In order to synthesize BMA031-specific oligomer probes for screening genomic clones, and to confirm that the correct genes had been cloned, the $V_H$ and $V_L$ regions of BMA 031 poly $A^+$ mRNA was sequenced. Nucleotide sequences of approximately 200 bases of the V regions of H and L chains were obtained from the initial sequencing reaction. Additional primers were synthesized based on this sequence in order to obtain the complete sequence. Probes corresponding to CDR3 of both the L and H chains were synthesized and used to screen genomic libraries (see Fig. 2).

*Isolation of VH and VL regions of BMA 031.* A genomic library of BMA 031 DNA was prepared in λ-phage EMBL-3. The library, $2 \times 10^6$ phage, was screened initially with the fragment probes HPH and HPL. The BMA 031 V region-specific oligomer probes were used in subsequent rounds of rescreening to isolate pure plaques. Four H chain clones and seven L chain clones were identified which hybridized strongly to both the fragment and BMA 031-specific oligomer probes. Southern analysis showed that the H chain clones contained the expected 5.6-kb *Eco*RI fragment and that the L chain clones contained the 3.0 kb *Hin*dIII fragment (see Fig. 1). The 3.0-kb *Hin*dIII $V_L$ fragment and the 5.6-kb *Eco*RI $V_H$ fragment were subcloned into pUC19.

*DNA sequencing of the $V_H$ and $V_L$ regions of BMA 031.* The 1.1-kb *Hin*dIII $V_H$ fragment and the 1.4 kb *Eco*RI/*Hin*cII $V_L$ fragment were subcloned into pUC19 and sequenced directly by the dideoxy method. The coding sequences are shown in Figure 2. The positions of the signal sequences, CDR, and oligomer probes are indicated. Analysis of the sequences indicates that BMA 031 $V_H$ is derived from the $JH_3$ minigene and is a member of subgroup IIB (36). It was also found that the D segment in BMA 031 is derived from DSP 2.2. The sequencing also demonstrated that BMA 031 $V_L$ is derived from the $JK_5$ minigene and belongs to κ subgroup VI.

*Construction and expression of chimeric BMA 031 H and L chain genes.* The 5.6-kb *Eco*RI BMA 031 VH fragment was cloned into the *Eco*RI site of mammalian expression vectors which contain either the human gamma-1 or gamma-4 C region and the *gpt* gene (Fig. 3*A*). The 3.0-kb *Hin*dIII BMA 031 VL fragment was cloned into the *Hin*dIII site of a similar vector that contains the human κ C region and the *neo* gene (Fig. 3*B*). The two plasmids were co-transfected into Sp2/0-Ag14, a non-Ig-producing murine hybridoma, by electroporation. Transfection efficiency was approximately $1 \times 10^{-5}$. After 2 wk of drug selection, culture supernatants were assayed for the presence of chimeric antibodies produced by the transfectomas.

*Analysis of chimeric BMA 031 transfectomas.* Culture supernatants from drug-resistant cells were assayed for the presence of murine/human chimeric antibody by ELISA. The antibody used to coat the microtiter plates was goat anti-human IgG (Fc specific) and goat anti-human κ antibody conjugated to horseradish peroxidase was used to detect Ag-antibody complexes. The 10 highest producing transfectomas from each transfection ($\gamma 1$ and $\gamma 4$) were assayed for antibody production daily for 1 wk. Chimeric antibody was produced at a rate of 1.2 to 7.0 pg/cell/24 h (data not shown). None of the chimeric antibodies reacted with goat anti-mouse antibody. The best clone with respect to secretion level and growth characteristics from each transfection was subcloned and expanded for further study. The clones are referred to as BMA 031-G1 (human IgG1 chimeric) and BMA 031-



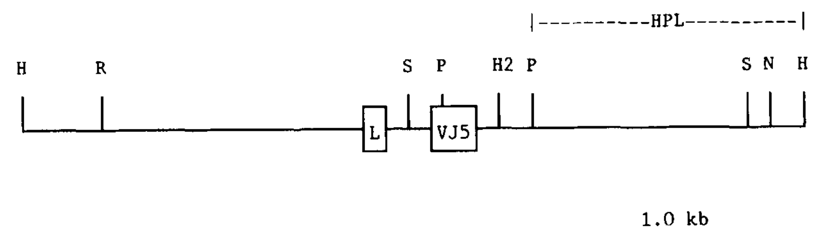




*Figure 1.* Partial restriction enzyme maps of BMA 031 V regions. *A*, the 5.6-kb *Eco*RI fragment containing the $VDJ_3$ exon; *B*, the 3.0 kb *Hin*dIII fragment containing the $VJ_5$ exon. *H*, *Hin*dIII; *H2*, *Hin*cII; *N*, *Nsi*I; *P*, *Pst*I; *R*, *Eco*RI; *S*, *Sau*I.

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A. BMA 031 VH

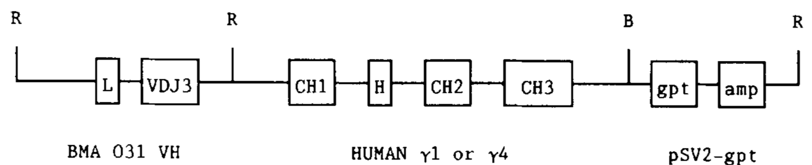
```
CTAACCATGGAATGGAGTTGGATATTTCTCTTTCTCCTGTCAGGAACTGCAGGTAAGGGG
      M  E  W  S  W  I  F  L  F  L  L  S  G  T  A
     |--------------Signal Sequence-----------------

CTCACCAGTTCAGTCAAATCTGAAGTGGAGACACAGGACCTGAGGTGACAATGACATCTA

CTCTGACATTCTCTCCTCAGGTGTCCACTCTGAGGTCCAGCTGCAGCAGTCTGGACCTGA
                      G  V  H  S  E  V  Q  L  Q  Q  S  G  P  E
                      ----------| 1

GCTGGTAAAGCCTGGGGCTTCAGTGAAGATGTCCTGCAAGGCTTCTGGATATAAATTCAC
 L  V  K  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  K  F  T

TAGCTATGTTATGCACTGGGTGAAGCAGAAGCCTGGGCAGGGCCTTGAGTGGATTGGATA
 S  Y  V  M  H  W  V  K  Q  K  P  G  Q  G  L  E  W  I  G  Y
|----CDR 1----|                                            |--

TATTAATCCTTACAATGATGTTACTAAGTACAATGAGAAGTTCAAAGGCAAGGCCACACT
 I  N  P  Y  N  D  V  T  K  Y  N  E  K  F  K  G  K  A  T  L
--------------------CDR 2----------------------|

GACTTCAGACAAATCCTCCAGTACAGCCTACATGGAGCTCAGCAGCCTGACCTCTGAGGA
 T  S  D  K  S  S  S  T  A  Y  M  E  L  S  S  L  T  S  E  D

                                *-----VH Probe-----*
CTCTGCGGTCCATTACTGTGCAAGAGGGAGCTACTATGATTACGACGGGTTTGTTTACTG
 S  A  V  H  Y  C  A  R  G  S  Y  Y  D  Y  D  G  F  V  Y  W
                           |------------CDR 3--------------|

GGGCCAAGGGACTCTGGTCACTGTCTCTGCAGGTGAGTCCTAACTTCTCCCATTCTAAAT
 G  Q  G  T  L  V  T  V  S  A
                JH3
```

B. BMA 031 VL

```
AAAATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGGTAACAGA
   M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S
  |----------------Signal Sequence------------------

GGGCAGGGAATTTGAGATCAGAATACAACCAAAATTATTTTCCCTGGGGAATTTGTGTCC

AAAATACAGTTTTTTCTTTTTCTTTTATCTAAATGTTGGGTGGTATAAAATTATTTTTTA

TCTCTATTTCTACTAATCCCTCTCTCTTTTTTGCTTTTTTCTAGTCATAATATCCAGAGG
                                                V  I  I  S  R  G
                                                ----------------|

ACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCAC
 Q  I  V  L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T
 1

CATGACCTGCAGTGCCACCTCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGTCAGG
 M  T  C  S  A  T  S  S  V  S  Y  M  H  W  Y  Q  Q  K  S  G
          |------------CDR 1-----------|

CACCTCCCCCAAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCTGCTCG
 T  S  P  K  R  W  I  Y  D  T  S  K  L  A  S  G  V  P  A  R
                           |-------CDR 2-------|

CTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGCATGGAGGCTGA
 F  S  G  S  G  S  G  T  S  Y  S  L  T  I  S  S  M  E  A  E

                            *-----VL Probe-----*
AGATGCTGCCACTTATTACTGCCAGCAGTGGAGTAGTAACCCGCTCACGTTCGGTGCTGG
 D  A  A  T  Y  Y  C  Q  Q  W  S  S  N  P  L  T  F  G  A  G
                        |----------CDR 3----------|     JK5

GACCAAGCTGGAGCTGAAACGTAAGTACACTTTTCTCATCTTTTTTTATGTGTAAGACAC
 T  K  L  E  L  K
```

*Figure 2.* DNA sequences encoding the V exons of BMA 031. *A*, the BMA 031 VH coding sequence; *B*, the BMA 031 VL coding sequence, each showing the signal sequence, start of the mature protein, the CDR, the J region and the V-specific probe used to isolate the V regions.

A. Heavy Chain




B. Light Chain

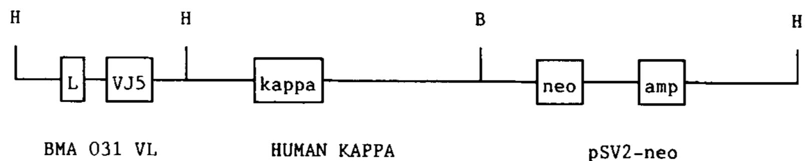



*Figure 3.* Expression vectors for chimeric Ig genes. *A*, the H chain expression vectors containing the BMA 031 VH region, the human $\gamma 1$ or $\gamma 4$ C region and the guanine phosphoribosyl transferase gene for selection. *B*, the L chain expression vector containing the BMA 031 VL region, the human $\kappa$ C region, and the neomycin resistance gene for selection.

G4 (human IgG4 chimeric).

*Purification and characterization of chimeric BMA 031 antibodies.* The antibodies secreted by BMA 031-G1 and BMA 031-G4 were tested to ensure that they were indeed BMA 031 mouse/human chimeric antibodies. A series of ELISA assays showed that the antibodies contain human $\kappa$ and $\gamma$ C regions. Moreover, the antibodies did not react with antibodies directed against murine $\kappa$ or $\gamma$ C regions. Isotyping reagents also confirmed that the chimeric antibodies were of the IgG1 and IgG4 isotypes (data not shown).

The BMA 031-G1 and BMA 031-G4 transfectomas were expanded and supernatant was collected from each line. Saturated cultures accumulate antibody to at least 35 $\mu$g/ml for BMA 031-G1 and 15 $\mu$g/ml for BMA 031-G4. The chimeric antibodies were partially purified by protein A Sepharose column chromatography by using a linear pH gradient for elution (100 mM sodium citrate, pH 3 to 7).

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Analysis of the antibodies by reducing and nonreducing SDS-PAGE showed a high degree of purity; although both chimeric antibodies showed some aggregation (data not shown). Also, BMA 031-G4 appeared to be more sensitive to reduction than BMA 031-G1, forming half-molecules in the presence of trace amounts of 2-ME.

*Specificity and affinity of BMA 031-G1 and BMA 031-G4.* The murine BMA 031 antibody is known to react with the $\alpha/\beta$-chains of the human TCR (37). Because the correct specificity of an antibody is essential for all functional analyses, it was important to establish that both chimeric antibodies have an identical specificity as murine BMA 031. In indirect immunofluorescence assays, crude culture supernatants or purified protein of BMA 031-G1 and BMA 031-G4 bind to PBL to the same extent as murine BMA 031. In addition, when using either purified T cells ($E^+$ cells) or directly labeled antibodies, both chimeric antibodies possess an identical specificity as compared with murine BMA 031 (data not shown).

The relative affinities of the BMA 031 antibodies were compared by competitive immunofluorescence assays. For this purpose, human PBL were preincubated with various concentrations of either BMA 031, BMA 031-G1, or BMA 031-G4. In a second incubation step, cells were stained with BMA 031-FITC and analyzed in a cell sorter. The data shown in Figure 4*A* indicate that murine and chimeric BMA 031 antibodies block the binding of BMA 031-FITC in the same dose-dependent manner. If either BMA 031-G1-FITC or BMA 031-G4-FITC was used as second-step reagent, identical results were obtained (Fig. 4, *B* and *C*). These data clearly demonstrate that murine and chimeric BMA 031 antibodies have very similar relative affinities.

*T cell activation by anti-CD3 and BMA 031 antibodies.* Previous studies with BMA 031 and anti-CD3 antibodies have shown differences in their abilities to activate T cells because of differences in both the Fc and Ag specificity. It is of interest, therefore, to analyze T cell activation by chimeric BMA 031 mAb, which have both the specificity of BMA 031 and the capacity to interact with human FcR. In Figure 5, data of a representative experiment are shown. In a 3-day proliferation assay (Fig. 5*A*), BMA 030 (anti-CD3) induced a typical bell-shaped curve. BMA 031-G1 and BMA 031-G4 need higher antibody concentrations, as compared with BMA 030, for T cell stimulation, but are much more mitogenic than BMA 031. Because the BMA 031 antibodies differ only in the C region of the molecule, these differences in T cell activation must be attributed to Fc-mediated functions. Interestingly, with the BMA 031 chimeric antibodies, no high dose suppression effects were seen even at concentrations of up to 10 $\mu$g/ml. In principal, similar results should be obtained in a 6-day proliferation assay. However, BMA 031 triggers a much stronger proliferation at 6 days than at 3 days (Fig. 5*B*). Again, the chimeric antibodies stimulate T cells to a greater extent than BMA 031 and without high dose suppression. These data suggest that BMA 031 triggers T cell proliferation in a more Fc-independent fashion, whereas the chimeric antibodies are able to provide an additional stimulatory signal derived from accessory cells.

To address this hypothesis, we carried out proliferation experiments with purified T cells as well as with $F(ab')_2$ fragments of mAb. The data of two representative experiments, shown in Table I, demonstrate that under experimental conditions in which anti-CD3 mAb BMA 030 is unable to stimulate T cell proliferation caused by the inability of Fc-mediated cross-linking (absence of accessory cells in the CD7 population or use of $F(ab')_2$ fragments), the stimulatory capacity of BMA 031, BMA 031-G1, or BMA 031-G4 is not impaired. These data strongly support the idea that, besides Fc-mediated reactions, the specificity of BMA 031 is instrumental for the differences in T cell activation as compared with anti-CD3 mAb.

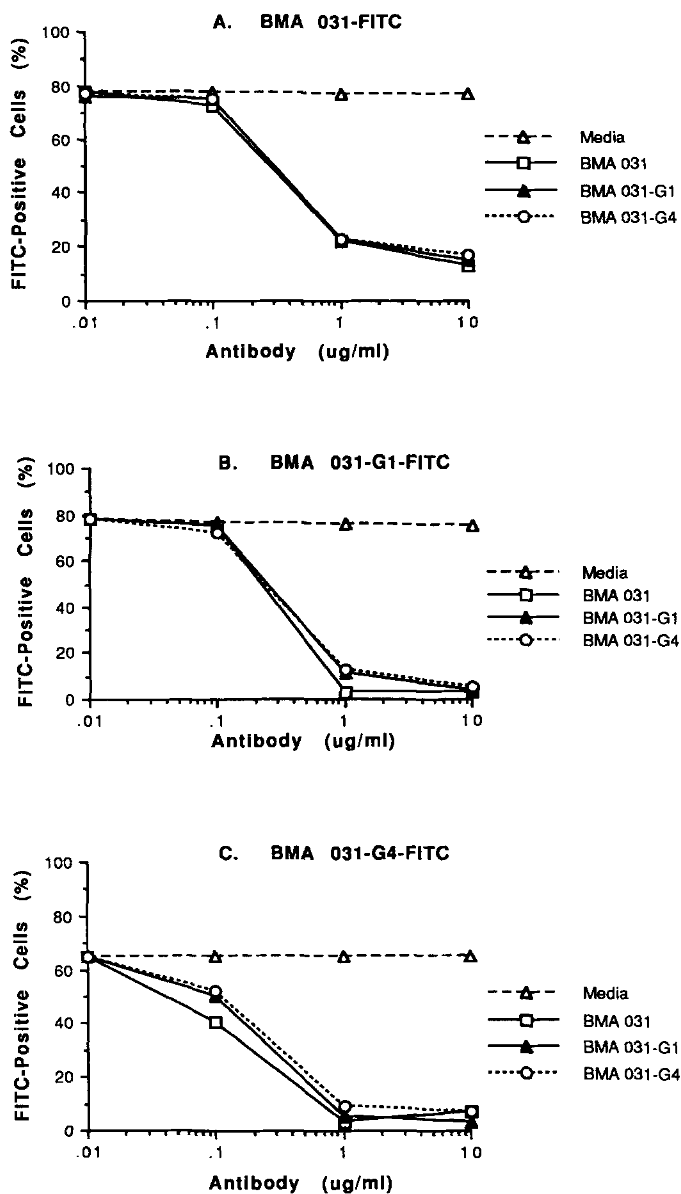


*Figure 4.* Relative affinities of BMA 031 antibodies. Competitive immunofluorescence assays with the BMA 031 antibodies, HPB mononuclear cells, and FITC-BMA 031 antibodies (10 $\mu$g/ml) were performed as outlined under *Materials and Methods*. *A*, BMA 031-FITC; *B*, BMA 031-G1-FITC; *C*, BMA 031-G4-FITC.

*Cell cytotoxicity with BMA 031-G1 and BMA 031-G4.* Murine mAb are usually not very effective in mediating cytolysis in either ADCC or CDC assays. In both cases, the Fc part of the molecule either does not allow optimal activation of the C cascade or provides an inappropriate interaction with human killer cells. Therefore, it was of interest to see if the human Fc part of the chimeric BMA 031 antibodies could alter the cytolytic capacity demon-

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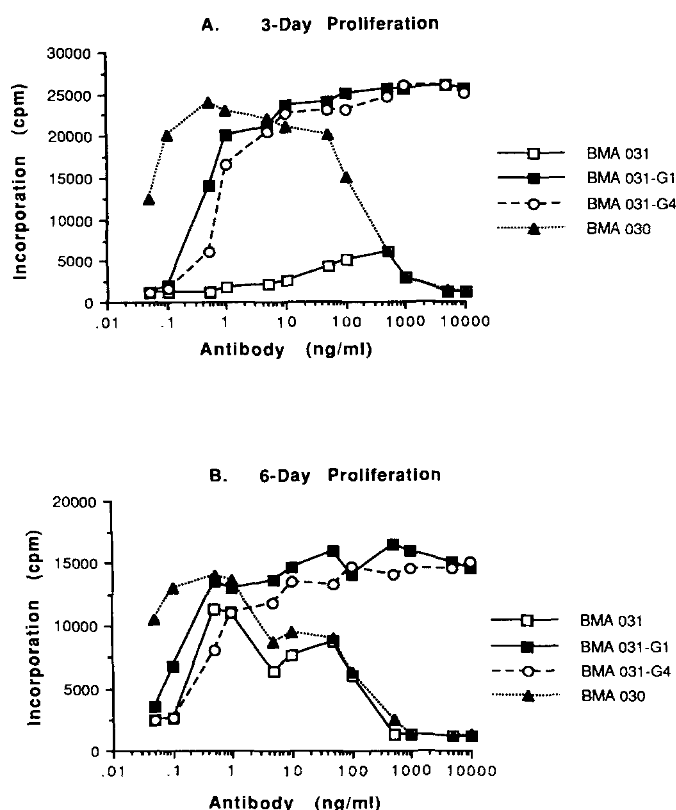


*Figure 5.* T cell activation by anti-CD3 and BMA 031 antibodies. *A*, a 3-day lymphocyte proliferation assay; *B*, a 6-day lymphocyte proliferation assay. Experimental conditions were as described under *Materials and Methods*. Medium and phytohemagglutinin controls (*A*) were 750 cpm and 24,500 cpm, respectively. Medium and splenic adherent cell controls (*B*) were 3,000 cpm and 12,500 cpm, respectively.

TABLE I
*Induction of T cell proliferation*

| T Cell Activation by[a] | $[^{14}C]$TdR Incorporation (cpm[b]) | | |
|---|---|---|---|
| | Experiment 1 | Experiment 2 | |
| | n-PBL[c] | n-PBL | $CD7^+$ cells[d] |
| BMA 030 | 34,765 ± 5% | 24,910 ± 10% | 225 ± 30% |
| BMA 030-$F(ab')_2$ | 720 ± 3% | 445 ± 8% | 460 ± 28% |
| BMA 031 | 3,415 ± 1% | 4,840 ± 15% | 1,375 ± 30% |
| BMA 031-G1-$F(ab')_2$ | 2,635 ± 10% | 3,485 ± 18% | 4,540 ± 8% |
| BMA 031-G4-$F(ab')_2$ | 4,032 ± 4% | 7,360 ± 15% | 5,660 ± 30% |
| Medium | 170 ± 15% | 300 ± 20% | 150 ± 30% |

[a] Concentrations used were 1 ng/ml for BMA 030 and 10 μg/ml for the BMA 031 antibodies.
[b] Mean of four values in a 3-day proliferation assay.
[c] Ficoll-separated PBL.
[d] Isolated by sorting (FACstar plus).

strated by BMA 031.

In CDC assays, only murine BMA 031 was able to lyse peripheral blood T cells, provided a selected rabbit C batch was used. The titer is strongly dependent on the blood donor and C batch and can vary from 1/200 to 1/6000 (calculated for 1 mg protein/ml). When other C sources (guinea pig or human) or HPB-ALL target cells were used, no CDC could be detected with BMA 031. The chimeric antibodies were unable to mediate CDC under any of the conditions tested (data not shown).

Because the chimeric BMA 031 antibodies were able to interact efficiently with human FcR in the T cell proliferation assays, there was a strong possibility that they would have high ADCC titers as well. To evaluate the ADCC capacity of these mAb, we compared them with rabbit anti-GH-1 antiserum. This antiserum was the best out of eight rabbit anti-human T cell globulins in ADCC capacity. The data of a representative experiment are shown in Figure 6. Even at low E:T ratios (Fig. 6*A*) or extremely low antibody concentrations (Fig. 6, *B* and *C*), chimeric BMA 031 antibodies are highly potent in killing HPB-ALL cells. In contrast, murine BMA 031 is very poor at ADCC. Further experiments will be needed to identify, in more detail, the effector cells capable of interacting with BMA 031-G1 and BMA 031-G4.

## DISCUSSION

We have joined the DNA segments encoding the murine VH and VL exons from the BMA031 mAb specific for the human TCR to the DNA segments encoding human $\gamma$-1 or $\gamma$-4 and $\kappa$C regions. When the chimeric genes were introduced into non-Ig producing Sp2/0 cells, functional chimeric antibodies with an identical affinity and specificity as murine BMA 031 were assembled and secreted.

The biologic activities of the chimeric antibodies are clearly different from those of BMA 031. Both BMA 031-G1 and BMA 031-G4 stimulate human T cells to a much greater extent than BMA 031 and without high dose suppression. Murine anti-CD3 mAb of the IgG2a isotype, such as BMA 030 and OKT3, are known to be highly mitogenic for human T cells (26). For the induction of T cell proliferation via CD3 Ag, binding of mAb to the Ag must be followed by cross-linking of the Ig molecules. Under physiologic conditions, this is achieved by interaction of the Fc portion of the mAb with FcR on accessory cells. An additional signal derived from the accessory cells seems to be essential for complete T cell activation (26). The strength of anti-CD3-induced T cell proliferation is typically dose dependent, resulting in a bell-shaped dose response curve. As reported earlier (4), induction of T cell proliferation by BMA 031 is relatively weak and requires much higher antibody concentrations for a longer period of time for maximal stimulation. This low mitogenicity was ascribed predominantly to deficient interaction with human FcR (4, 10, 26). Recent studies, however, have suggested that anti-CD3 mAb and BMA 031 trigger T cells by different pathways (38). In our present studies, the chimeric BMA 031 antibodies have proved to be a powerful tool in discriminating between specificity and Fc-related contributions to signals involved in T cell activation. Our data strongly supports the hypothesis that the differences in T cell activation exhibited by BMA 031 as compared with anti-CD3 mAb is caused by differences in specificity as well as Fc-mediated reactions. Under physiologic conditions, in T cell activation mediated by chimeric BMA 031 antibodies, the activation signal derived from mAb binding to the TCR is superimposed on Fc-mediated trigger signals derived from accessory cells, whereas murine BMA 031 will deliver only the first signal. With chimeric BMA 031-$F(ab')_2$ fragments or accessory cell depleted T cells ($CD7^+$ cells), we could show, that in contrast to anti-CD3 mAb, BMA031 is able to trigger T cell proliferation in an Fc-independent way. Experiments with PBL, $E^+$ cells, and thymocytes, where accessory cell function was bypassed by co-stimulation with an anti-CD28 mAb, corroborate this Fc independence (39). In addition, some differences in T cell activation between anti-CD3 and BMA 031 mAb, such as triggering of $Ca^{2+}$ influx, could only be explained by differences in specificity as well (13). Experiments are

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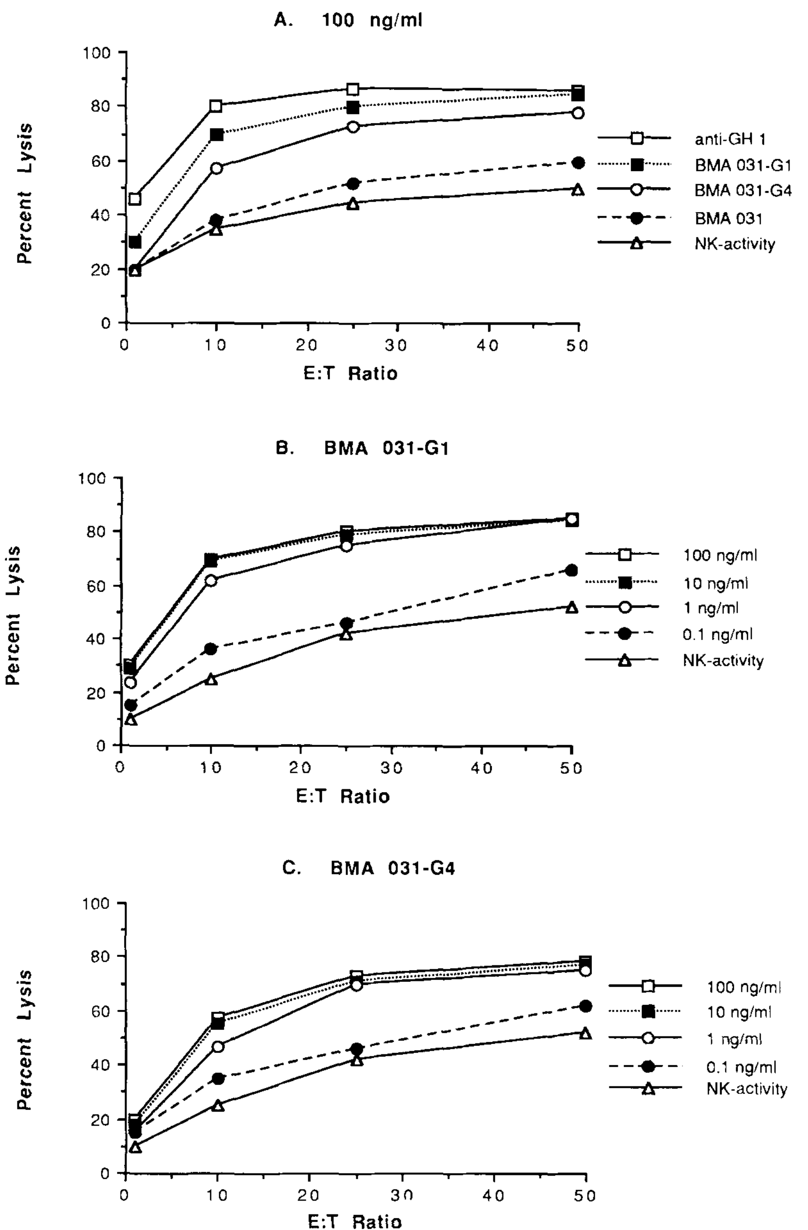


*Figure 6.* ADCC capacity of BMA 031 antibodies. The cytolytic capacity of the BMA 031 antibodies was determined in a 20-h $^{51}Cr$ release assay as described under *Materials and Methods*. *A*, lysis in the presence (ADCC) or absence (NK activity) of antibody (100 ng/ml); *B*, lysis at various concentrations of BMA 031-G1; *C*, lysis at various concentrations of BMA 031-G4.

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in progress to identify the responding T cell population. Similar results have been shown with experiments in the murine system with anti-murine CD3 and anti-murine TCR-$\alpha/\beta$ mAb. On distinct T cell subpopulations, both mAb differ in the quality of signals transduced (9). Because incomplete T cell activation of selective T cell subpopulations may lead to anergy or apoptosis (40, 41), selective triggering of T cells by BMA 031 might be predominantly responsible for its immunoregulatory effects in vivo.

For therapeutic applications of mAb in T cell disorders, it may be advantageous to augment the cytolytic capacity of mAb. In particular, for treatment of T cell leukemias, a mAb capable of eliminating highly malignant tumor cells by means of the patients immune system might be superior to cytostatic drugs because only cells expressing a specific Ag are destroyed. Murine mAb are usually weak in cytolytic capacity and BMA 031 is no exception. Under various experimental conditions, only marginal cytolytic activity could be obtained with BMA 031. Compared with the strong ADCC activity achieved with selected polyclonal rabbit anti-T cell globulins (anti-GH1 antiserum), the ADCC activity obtained with different anti-leukocyte mAb, even of other specificities and isotypes, is weak. In contrast, both BMA 031-G1 and BMA 031-G4 are highly effective in cytolytic capacity. Even at low E:T ratios and extremely low antibody concentrations, the chimeric antibodies are highly potent in killing HPB-ALL cells.

The results presented suggest that chimeric BMA031 may have clinical utility in preventing transplant rejection, graft-vs-host disease, autoimmune diseases, and other T cell-related problems. The chimeric antibody retains the affinity and specificity of murine BMA 031 but contains human C regions, which should reduce or eliminate the patients immune response to the mouse C regions. Engineered BMA 031 antibodies with humanized V regions and human C regions have been produced recently (C. W. Shearman, D. P. Pollock, G. White, K. Hehir, G. P. Moore, E. J. Kanzy, and R. Kurrle, manuscript in preparation) and these should further reduce

the patients immune response during therapy. Moreover, effector functions associated with the human C regions are enhanced in vitro relative to murine BMA031 and may also be in vivo when the humanized antibodies are used for immunotherapy. We are now in a position to test the efficacy of humanized BMA031 in clinical trials.

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