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(54) Title: SILENSED ANTI-CD28 ANTIBODIES AND USE THEREOF

(57) Abstract: The present invention provides anti-CD28 antibodies which are defective in mitogenic activity (silenced anti-CD28 antibodies), methods of producing, compositions containing the antibody and methods of immunosuppression, inducing T-cell tolerance and treating organ and/or tissue transplant rejections.

TITLE OF THE INVENTION

SILENCED ANTI-CD28 ANTIBODIES AND USE THEREOF

Field of the Invention

This invention relates to anti-CD28 antibodies defective of mitogenic activity and to uses thereof.

Background of the Invention

Immune reactions, particularly organ transplant rejections, are chiefly attributed to the activation of T-lymphocytes. This activation of T cells is induced by a signal from antigen-presenting cells (APC). The signal from the APC involves a first signal via the T-cell receptor (TCR) and a second signal (costimulatory signal) via costimulatory molecules. The first signal is from the major histocompatibility antigen (MHC) complex of peptides antigen where the APC is presents the T-cell antigen through the TCR. The second signal is mediated by several co-stimulatory molecules, examples of which include B7 (B7-1 (CD80) and B7-2 (CD86)) are known as ligands on the APC side and CD28, CTLA-4, etc. as receptors on the T-cell side. The ligand B7 is a glycoprotein belonging to the immunoglobulin super family and is expressed in B cells etc. which belong to the antigen-presenting cell group. Both CD28 and CTLA-4, which recognize B7 as the common ligand, are transmembrane glycoproteins belonging to the immunoglobulin super family. Thus, the activation of T cells is regulated by the concurrent transduction of the first signal via TCR and the second signal from, e.g., the B7 and CD28/CTLA-4. The signal from B7-to CD28 is known to promote whereas the signal from B7 to CTLA-4 inhibits the activation of T cells [Waterhouse et al., Science, 270:985-988 (1995)].

Heretofore, for the purpose of inducing immunosuppression or tolerance, attempts have been made to block the B7-CD28 signal by administering CTLA-4Ig, anti-B7-1 antibody/anti-B7-2 antibody, anti-CD28 antibody or the like. For example, CTIA-4Ig binds to B7 thereby interfering with the reaction between B7 and CD28 and, as a consequence, block the signal from CD28 to exhibit immunosuppressive activity. However, since the reaction between B7 and CTLA-4 is also inhibited simultaneously, the signal of CTLA-4 acting negatively on the activation of T cells is also suppressed so that the desired tolerance is not induced (Kirk et al., Proc. Natl. Acad. Sci. USA, 94:8789-8794 (1997). An anti-B7 antibody was also prepared and reported to have suppressed activation of T cells but just as in the case of CTLA-4Ig, it suppressed the CTLA-4 signal as well.

An anti-CD28 antibody, in an *in vitro* experiment, was found to produce a mitogenic effect on T cells, and the combination of the stimulation with this antibody and an anti-CD3 antibody promoted the growth and activation of T cells and enhanced the production of cytokines [WO 90/05541, Eur. J. Immunology, 16, 1289-1296 (1986), etc.]. Furthermore, mitogenic stimulation of the CD28 receptor of the T cell by an anti-CD28 antibody has been stimulated *in vivo* resulted in the generation of a T-cell activation signal similar to the second signal from B7 to CD28 [Yin et al., J. Immunology, 163:4328-4334 (1999)]. These T-cell activating functions suggested that an anti-CD28 antibody might be used as an immunopotentiator in the therapy of cancer and AIDS (WO 90/05541).

SUMMARY OF THE INVENTION

The anti-CD28 antibodies prepared by conventional technologies exert a mitogenic action on T cells. Although the reasons for this mitogenic activity is not fully understood, the binding of the anti-CD28 Fc region to the Fc receptor of the antigen-presenting cell is believed to be the probable reason (Cole et al., J. Immunology, 36:159 (1997). Therefore, by utilizing genetic engineering technology, we introduced mutations into the Fc receptor binding site of the anti-CD28 antibody to modify the antibody so that it would no longer have mitogenic activity. One such antibody the present inventors have generated, TN228 IgG2M3, in which IgG2M3 has two amino acid substitution in IgG gene. Furthermore, we demonstrated that the resulting silenced anti-CD28 antibody has no mitogenic activity which is very useful for inducing T cell tolerance.

Therefore, the present invention provides anti-CD28 antibodies having no mitogenic activity (hereinafter referred to as silenced anti-CD28 antibodies), and uses of such antibodies for the manufacture of medicaments for suppressing immune reactions, particularly transplant rejections, and inducing immunotolerance by using said antibodies.

An object of the present invention is a silenced anti-CD28 antibody, where the anti-CD28 antibody may be a chimeric antibody and or a humanized antibody. The variable regions of the anti-CD28 antibodies may include the amino acid sequences shown in SEQ ID NOS: 2, 4, 7 and 9 and polynucleotides encoding such amino acid sequences. For example, such polynucleotides include SEQ ID NOS: 1, 3, 6, and 8.

Another object of the present invention is vectors and cell hosts comprising the polynucleotides which encode the anti-CD28 antibodies.

Another object of the present invention is methods for producing the silenced anti-CD28 antibody by culturing a cell host comprising the polynucleotides which encode the anti-CD28 antibodies under conditions which allow expression of the polynucleotide and collecting the gene products produced.

Another object of the present invention is a pharmaceutical composition comprising one or more of the silenced anti-CD28 antibodies, preferably admixed with one or more pharmaceutically acceptable ingredients.

The silenced anti-CD28 antibodies may be useful for inducing T-cell tolerance, immunosuppression and as a prophylactic/therapeutic drug for organ or tissue transplant rejection. Accordingly, methods for inducing T-cell tolerance, immunosuppression, and providing a prophylaxis or treatment therapy during an organ or tissue transplant rejection by administering one or more of the silenced anti-CD28 antibodies to a mammal are also provided. Preferably, such silenced anti-CD28 antibodies are administered in as a pharmaceutical composition as described herein and may include additional drug/pharmaceuticals where appropriate.

The present invention also provides use of the silenced anti-CD28 antibodies for the manufacture of a medicament for inducing T-cell tolerance, providing immunosuppression and treating organ or tissue transplant rejection.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Plasmid constructs for ChTN228 antibody expression. VL and VH of murine TN228 were constructed as mini-exons flanked by Xbal sites. The VL sequence was incorporated into the expression vector pVk and the VH sequence was incorporated into the expression vector pVg2M3.

Figure 2. Nucleotide sequences and deduced amino acid sequences of the light chain of ChTN228 in the mini-exons. The signal peptide sequences are in italics. The CDRs are underlined. The mature light chain begins with an aspartic acid residue (bold letter). Untranslated and intron sequences are in lower case. (SEQ ID NOS: 1 and 2).

Figure 3. Nucleotide sequences and deduced amino acid sequences of the heavy chain variable regions of ChTN228 in the mini-exons. The signal peptide sequences are in italics. The CDRs are underlined. The mature heavy chain begins with a glutamine residue (bold letter). Untranslated and intron sequences are in lower case. (SEQ ID NOS: 3 and 4).

Figure 4. Competition experiment. P815/CD28$^+$ cells were incubated with 25 ng of MuTN228-FITC and two-fold serial dilutions of either ChTN228 or MUTN228 as described. P815/CD28' cells were also incubated with MuTN228-FITC alone, without any competitor. The mean channel fluorescence for each sample was plotted against the concentration of competitor.

Figure 5. Inhibition effect of TN228-IgG2m3 on human primary MLR(1). Percentage inhibition of primary MLR from four individuals were shown separately.

Figure 6. Inhibition effect of TN228-IgG2m3 on human primary MLR (2). Percentage inhibition of primary MLR from four individuals were shown separately.

Figure 7. The effect of TN228-IgG2m3 on secondary MLR.

The data from two volunteers were shown separately.

[^{3}H]-thymidine uptake in 2nd MLR were presented as percentage of dpm of Raji stimulation alone in lst MLR as 100. TN228-IgG2m3:0.lug/mL

Figure 8. Plasmid constructs for HuTN228 antibody expression. VL and VH of humanized TN228 were constructed as mini-exons flanked by XbaI sites. The VL sequence was incorporated into the expression vector pVk and the VH sequence was incorporated into the expression vector pVg2M3

Figure 9. Nucleotide sequences and deduced amino acid sequences of the heavy chain variable regions of HuTN228 in the mini exons. The signal peptide sequences are in italics. The CDRs are underlined. The mature heavy chain begins with a glutamine residue (bold letter). (SEQ ID NOS: 6 and 7)

Figure 10. Nucleotide sequences and deduced amino acid sequences of the light chain variable regions of HuTN228 in the mini exons. The signal peptide sequences are in italics. The CDRs are underlined. The mature light chain begins with an aspartic acid residue (bold letter). (SEQ ID NOS: 8 and 9)

Figure 11. FACS competition assay. The binding of FITC-labeled MuTN228 to P815/CD28' cells in the presence of various amounts of competitor MuTN228 or HuTN228 antibody was analyzed in a flow cytometry competition experiment as described in the examples.

Figure 12. ELISA competition assay. The binding of biotinylated MuTN228 to sCD28-Fc in the presence of various amounts of competitor MuTN228 or HuTN228 antibody was analyzed in an ELISA competition experiment as described in the examples.

Figure 13. I-125 competition assay. The binding of ^{125}I labeled MuTN228 to P815/CD28$^+$ cells in the presence of various amounts of competitor MuTN228 or HuTN228 antibody was analyzed in an ^{125}I labeled antibody competition experiment as described in the examples.

Figure 14. Plasmid constructs for PV1-IgG3 antibody expression. V_L and V_H of PV1 were constructed as mini-exons flanked by XbaI sites. The V_L sequence was incorporated into the expression vector pMVk.rg.dE, and the V_H sequence into the expression vector pMVg3.D.Tt. The two plasmids were then recombined to generate a single plasmid co-expressing the heavy and light chains of PV1-IgG3.

Figure 15A . Sequences of cDNA and deduced amino acid sequences of the light chain and heavy chain in the mini-exons. The CDRs are underlined. The mature light chain begins with an aspartic acid residue (double underlined) at position 20. (SEQ ID NOS: 10 and 11).

Figure 15B. Sequences of cDNA and deduced amino acid sequences of variable regions of PV1 in the mini-exons. The CDRs are underlined. The mature heavy chain with glutamine (double underlined) at position 20. (SEQ ID NOS: 12 and 13).

Figure 16. Analysis of PV-1-IgG3 by size exclusion chromatography using HPLC as described in *Methods*. The protein was monitored by its absorbance at 280 nM.

Figure 17. SDS-PAGE analysis of mouse IgG3 isotype control (lane 1), PV1 (lane 2), and PV1-IgG3 (lane 3). Proteins in Panel A were run under nonreducing conditions, and in Panel B reducing conditions. MW represents molecular weight markers. The numbers are MW standards in kD.

Figure 18. EL4 cells stained with PV1 (A), 37.51 (B), or PV1-IgG3 (C), and analyzed by flow cytometry. Secondary antibodies used were: FITC-conjugated donkey anti-Armenian hamster IgG (H+L) for PV1, FITC-conjugated donkey anti-Syrian hamster IgG for 37.51, and FITC-conjugated goat anti-mouse kappa for PV1-IgG3. The solid line profiles represent cells stained with secondary antibodies only. The broken line profiles represent cells stained with both primary and secondary antibodies as described in *Methods* . Mouse IgG3 isotype control did not stain EL4 cells (data not shown).

Figure 19. (A). Excess PV1, or PVI-IgG3 competes with R-PE-conjugated PV1 for binding to EL4 cells. Thin solid line (black) in flow cytometry histogram represents cells without any staining, thick solid line (dark blue) cells stained with R-PE-PV1 alone, thin broken line (magenta) cells stained with R-PE-PV1 and excess unconjugated PV1, and thin double broken line (light blue) cells stained with R-PE-PV1 and excess unconjugated PV1-IgG3. Excess mouse IgG3 isotype control had no effect on R-PE-PV1's binding to EL4 cells (data not shown). (B). Excess 145.2C11, or 145.2C11-IgG3 compete with R-PE-conjugated 145.2C11 for binding to EL4. Thin solid line (black) represents cells without any staining, thick solid line (dark blue) cells stained with R-PE-145.2C11 alone, thin broken line (magenta) cells stained with R-PE-145.2C11 and excess unconjugated 145.2C11, and thin double broken line (light blue) cells stained with R-PE-145.2C11 and excess unconjugated 145.2C11-IgG3. (C). Excess PV1 competes with PV1-IgG3 for binding to EL4 cells. EL4 cells were stained with PV1-IgG3 with or without excess PV1. Cells were washed and stained with mouse IgG3-specific, FITC-conjugated donkey anti-mouse IgG (H+L). Thin solid line (black) represents cells stained with secondary antibodies only, thick solid line (dark blue) cells stained with PV1-IgG3 and secondary antibodies, and thin broken line (magenta) cells stained with PV1-IgG3 and excess PV1, and secondary antibodies.

Figure 20. Mouse splenic cells stained with PV1-IgG3 and 145.2C11. Cells were stained with mouse IgG3 isotype control (A) or PV1-IgG3 (B), counter-stained with R-PE-conjugated goat anti-mouse IgG3 and

with FITC-conjugated 145.2C11, and analyzed by two-color flow cytometry as described in *Materials and Methods* . Only cells in the lymphocyte gate were analyzed. PV1-IgG3-positive cells are in the upper quadrants and CD3-positive cells are in the right side quadrants. The number in each quadrant represents percentage of the cells in that particular quadrant.

DETAILED DESCRIPTION OF THE INVENTION

In the context of this invention, the term "silenced anti-CD28 antibody" means any anti-CD28 antibody defective of mitogenic activity. More specifically, it is an antibody which binds specifically to the antigen CD28 receptor on the surface of the T cell and does not promote the growth or activation of T cells by combined stimulation with an anti-CD3 antibody.

A silenced anti-CD28 antibody can be constructed on the basis of an anti-CD28 antibody or an anti-CD28 antibody-producing hybridoma by mutating or modifying an agonistic anti-CD28 antibody by a genetic engineering technique or by chemical modification. Taking the use of genetic engineering technology as an example, the binding affinity of the anti-CD28 antibody for the Fc receptors can be reduced or eliminated by introducing a mutation into the amino acid sequence of the Fc domain of the antibody. For example, a silenced anti-CD28 antibody can be obtained by isolating cDNA from hybridoma cells capable of producing an anti-CD28 monoclonal antibody and introducing a mutation(s) into the region of the sequence corresponding to the Fc domain which plays an important role in the binding to the Fc receptor (WO 88/07089). The site of mutation is not particularly restricted inasmuch as the binding to the Fc receptors may be inhibited. Thus, in the case of a Class IgG antibody, for instance, the H-chain amino acid residues 234, 235, 236, 237, 318, 320 and 322 are preferred and a silenced anti-CD28 antibody can be constructed by replacing at least one of these amino acids with a different amino acid.

The source of such a silenced anti-CD28 antibody can be judiciously selected according to the target animal in which the antibody is used. For example, nonhuman monoclonal antibodies contain amino acid sequences showing antigenicity in humans over a fairly broad range. Many studies have shown that the immune response of a patient to a foreign antibody following injection of the antibody is remarkably intense and the very administration of the antibody may bring the patient into a perilous condition or deprive the antibody of the therapeutic utility. Therefore, it is recommendable to replace the Fc region so as to make the antibody relatively more homologous to the therapeutic target animal, replace the framework potions of the variable regions, or use the antibody obtained from a transgenic animal into which the antibody gene of the target animal has been

introduced. For example, when the antibody is to be administered to a human, a chimeric antibody (EP125023) available on replacement of the Fc region, a humanized antibody with the framework portion replaced (EP0239400, EP045126) or a human antibody (EP546073, WO 97/07671) obtained from a transgenic animal into which the human antibody gene has been introduced. By introducing mutations in these antibodies by genetic engineering techniques such as those described above or by chemical modification, the mitogenic activity of the antibodies can be reduced or eliminated.

As specific examples of the anti-CD28 antibody having a silenced Fc region, there can be mentioned not only the antibodies described hereinafter in the Examples section but also the antibodies synthetically prepared using the constant region gene of the therapeutic target animal and the variable region polynucleotides based on the amino acid sequences of variable regions shown in SEQ ID NO:2 and NO:4 or SEQ ID NO:7 and NO:9. Examples of such polynucleotides are SEQ ID NOS:1, 3, 6, and 8.

More specific examples of this invention are HuTN228 and MuTN228 and Fab fragments thereof F(ab)'2 fragments thereof, derivatives thereof and etc..

As appreciated by those skilled in the art, because of third base degeneracy, almost every amino acid can be represented by more than one triplet codon in a coding nucleotide sequence. Further, minor base pair changes may result in variation (conservative substitution) in the amino acid sequence encoded, are not expected to substantially alter the biological activity of the gene product. Thus, a nucleic acid sequencing encoding a protein or peptide as disclosed herein, may be modified slightly in sequence (e.g., substitution of a nucleotide in a triplet codon), and yet still encode its respective gene product of the same amino acid sequence.

The term "expression vector" refers to an polynucleotide which encodes the peptide of the invention and provides the sequences necessary for its expression in the selected host cell. Expression vectors will generally include a transcriptional promoter and terminator, or will provide for incorporation adjacent to an endogenous promoter. Expression vectors will usually be plasmids, further comprising an origin of replication and one or more selectable markers. However, expression vectors may alternatively be viral recombinants designed to infect the host, or integrating vectors designed to integrate at a preferred site within the host's genome. Examples of expression vectors are disclosed in Molecular Cloning: A Laboratory Manual, Second Edition, Sambrook, Fritsch, and Maniatis, Cold Spring Harbor Laboratory Press, 1989.

Suitable host cells for expression of the silenced anti-CD28 antibody include prokaryotes, yeast, archae, and other eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are well known in the art, *e.g.*, Pouwels *et al.* Cloning Vectors: A

Laboratory Manual, Elsevier, New York (1985). Preferably, the cells are mammalian cells. The vector may be a plasmid vector, a single or double-stranded phage vector, or a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The vectors, in the case of phage and viral vectors also may be and preferably are introduced into cells as packaged or encapsulated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells. Cell-free translation systems could also be employed to produce the proteins using RNAs derived from the present DNA constructs.

The silenced anti-CD28 antibodys/proteins can be purified by isolation/purification methods for proteins generally known in the field of protein chemistry. More particularly, there can be mentioned, for example, extraction, recrystallization, salting out with ammonium sulfate, sodium sulfate, etc., centrifugation, dialysis, ultrafiltration, adsorption chromatography, ion exchange chromatography, hydrophobic chromatography, normal phase chromatography, reversed-phase chromatography, gel filtration method, gel permeation chromatography, affinity chromatography, electrophoresis, countercurrent distribution, etc. and combinations of these.

According to the present invention, purified antibodies may be produced by the recombinant expression systems described above. The method comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes the protein under conditions sufficient to promote expression of the protein. The protein is then recovered from culture medium or cell extracts, depending upon the expression system employed. As is known to the skilled artisan, procedures for purifying a recombinant protein will vary according to such factors as the type of host cells employed and whether or not the recombinant protein is secreted into the culture medium.

The silenced anti-CD28 antibody when formulated into a pharmaceutical composition can be used in (a) transplant rejections following the transplantation of organs or tissues, such as heart, kidney, liver, bone marrow, skin, cornea, lung, pancreas, small intestine, muscle, nerve, etc.; (b) graft-versus-host reactions in the transplantation of bone marrow; (c) autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, type I diabetes, etc.; and (d) immune diseases such as asthma, atopic dermatitis, etc.

While the silenced anti-CD28 antibody by itself can be expected to suppress immune reactions and transplant rejections and induce immunotolerance, it can also be used in combination with other drugs. Among

such other drugs which are useful for combining with the silence anti-CD28 antibody are various immunosuppressants such as rapamycin, deoxyspergaulin, anti-CD40 antibody, anti-CD40L antibody, prograf, cyclosporin A, anti-IL-2 antibody, anti-IL-2 receptor antibody, anti-IL-12 antibody, anti-IL12 receptor antibody and MMF. Rapamycin, in particular, inhibits transduction of the signal related to growth of T cells among signals from the IL2 receptor but does not inhibit transduction of the apoptosis-related signal, so that its use in combination with a specific inhibitor of the CD28 signal is expected to be useful.

The silenced anti-CD28 antibody of this invention can be administered orally or parenterally, preferably by the intravenous, intramuscular or subcutaneous route.

The silenced anti-CD28 antibody of this invention can be prepared in the form of a solution or a lyophilized powder and, where necessary, may be formulated with various pharmaceutically acceptable additives such as an excipient, diluent, stabilizer, isotonizing agent and buffer. The preferred additives include a sugar such as maltose, a surfactant such as polysorbate, an amino acid such as glycine, a protein such as human serum albumin, and a salt such as sodium chloride.

Also, the dosage form such as injectable preparations (solutions, suspensions, emulsions, solids to be dissolved when used, etc.), tablets, capsules, granules, powders, liquids, liposome inclusions, ointments, gels, external powders, sprays, inhalating powders, eye drops, eye ointments, suppositories, pessaries, and the like can be selected appropriately depending on the administration method, and the peptide of the present invention can be accordingly formulated. Formulation in general is described in Chapter 25.2 of Comprehensive Medicinal Chemistry, Volume 5, Editor Hansch et al, Pergamon Press 1990.

The dosage of the pharmaceutical composition of this invention is dependent on the specific composition, the type of disease as the target of therapy or prophylaxis, the method of administration, the patient's age and condition and the duration of treatment, among other variables. However, in the case of intravenous, intramuscular or subcutaneous administration, 0.01-100 mg/kg, preferably 0.1-10 mg/kg, per day per adult can be administered.

When the silenced anti-CD28 antibody of this invention is used for suppression of transplant rejection or induction of immunotolerance in an organ or tissue transplantation, the composition can be administered in a dose of about 1 mg/kg/day immediately before transplantation, immediately after transplantation, and 3, 7, 12, 18, 25, 35, 45 and 60 days after transplantation, by intravenous, intramuscular or subcutaneous injection. The administration frequency and dosage may be judiciously increased or decreased while the course of rejection reaction after transplantation is monitored.

While the administration interval depends on the method of administration used and the patient's condition, among other factors, not only continuous administration but also intermittent administration is feasible. Thus, since the silenced anti-CD28 antibody of this invention is an antibody, it provides a sustained effect so that intermittent dosing may be rewarded with the expected efficacy. As to the period of treatment, once a tolerant state is established, this tolerance can be maintained even if the use of the silenced anti-CD28 antibody is discontinued. In this respect, this silenced anti-CD28 antibody is undoubtedly superior to other immunosuppressants the immunosuppressive effect of which declines after discontinuation.

EXAMPLES

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified. The examples below are carried out using standard techniques, that are well known and routine to those of skill in the art, except where otherwise described in detail.

Example 1. Amino acid sequencing of the mouse anti-human CD28 antibody

The hybridoma producing anti-human CD28 antibody (clone:TN228, mouse IgG1 kappa) was generously provided by Dr. Yagita (Juntendo University School of Medicine, Japan). Approximately 0.2 mg of purified anti-human CD28 antibody (TN228) was reduced in 0.64 M guanidine-HCl, 0.28 M Tris-HCl, pH 8.5, 0.055 M DT7 for 90' at 60 C (under argon), carboxymethylated by addition of iodoacetic acid to 0.13 M for 45' at room temperature (in the dark), followed by addition of DTr to 0.32 M (to terminate the carboxymethylation reaction), and immediately buffer-exchanged in 0.1 M sodium phosphate, 0.002 M EDTA, pH 8.0 using a PD-10 column (catalog #17-0851-01, Amersham Pharmacia Biotech, Uppsala, Sweden). The eluate was adjusted to 0.005 M DTT, 0.02 % glycerol, and one third of the solution (about 0.35 ml) was transferred to a separate tube for N-terminal deblocking of the heavy chain. The sample was digested with 1800 μU of pyroglutamate aminopeptidase (catalog # 7334, Takara Shuzo Co., Ltd., Tokyo, Japan) for 24 hours at 45 C. The N-terminal sequences of the light and heavy chains from the deblocked sample were determined by 20 cycles of automated Edman degradation and PTH analysis on a Model 241 Protein Sequencer (Hewlett Packard, Palo Alto, CA). The PTH derivatives were analyzed on a Hypersil ODS C18 column. The sequencer and HPLC were operated according to the manufacturer's instructions using reagents, solvents, and columns obtained from Hewlett Packard.

N-terminal sequencing results for TN228 deblocked with pyroglutamate aminopeptidase were as follows:

residue no.	amino acid	residueno.	amino acid	residueno.	amino acid	residue no.	amino acid
1	D, Q	6	Q, E	11	L	16	G, Q
2	I, v	7	s	12	A, V	17	Q, S
3	V, Q	8	P, G	13	V, A	18	R, L
4	L	9	A, P	14	S, P	19	A, S
5	T, K	10	S, G	15	L, S	20	T, I

Example 2. Cloning of variable region cDNAs

The V region cDNAs for the light and heavy chains of TN228 were cloned from the hybridoma cells by an anchored polymerase chain reaction (PCR) method described by Co et al. (Co, M.S., N.M. Avadalovic, P.C. Caron, M.V. Avadalovic, D.A. Scheinberg, and C. Queen. 1992. Chimeric and humanized antibodies with specificity for the CD33 antigen. J. Immunol. 148: 1149-1154.). Amplification was performed on cDNA using 3' primers that anneal respectively to the mouse kappa and gamma chain C regions, and a 5' primer that anneals to the added G-tail of the cDNA. For VL PCR, the 3' primer has the sequence (SEQ ID NO:14):

5' TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC 3'

with residues 17- 46 hybridizing to the mouse C_k region. For VH PCR, the 3' primers have the degenerate sequences (SEQ ID NOS: 15, 16, and 17):

5' TATAGAGCTCAAGCTTCCAGTGGATAGACCGATGGGGCTGTCGTTTTGGC 3'
(A / T at position 30; G at position 31; T at position 32)

with residues 17 - 50 hybridizing to mouse IgG C_H1. The non-hybridizing sequences in the two primer sets contain restriction sites used for cloning. The VL and VH cDNAs were subcloned into a TOPOII Blunt vector (Invitrogen, Inc., Carlsbad, CA) for sequence determination.

Several light and heavy chain clones were sequenced from two independent PCR reactions. For the light chain, two unique sequences homologous to mouse light chain variable regions were identified. One VL

sequence was non-functional due to a frame shift mutation and was identified as the non-productive allele. The other VL sequence was typical of a functional mouse kappa chain variable region. For the heavy chain, a unique sequence homologous to a typical mouse heavy chain variable region was identified. Their nucleotide sequences and their deduced amino acid sequences of variable region are described in Fig. 2 and Fig. 3.

Example 3. Construction and expression of chimeric TN228-IgG2M3

(Methods)

TN228 V_L and V_H were converted by PCR into mini-exon segments flanked by XbaI sites as described by He et al. (He, X.Y., Z. Xu, J. Melrose, A. Mullowney, M. Vasquez, C. Queen, V. Vexler, C. Klingbeil, M.S. Co, and E.L. Berg. 1998. Humanization and pharmacokinetics of a monoclonal antibody with specificity for both E- and P-selectin. J. Immunol. 160: 1029-1035) and were subcloned into the light chain and heavy chain expression plasmids (Fig. 1). Each mini-exon contains a signal peptide sequence, a mature variable region sequence and a splicing donor sequence derived from the most homologous mouse J chain gene. Such splicing donor sequences are used to splice the V region exon to the human antibody constant region. Each mini-exon was sequenced after it had been cloned into the expression vector to ensure the correct sequence was obtained and that no PCR errors were generated. The constant region exons of the light and heavy chain expression plasmids were also confirmed by sequencing.

In this specification, ChTN228 refers to a chimeric antibody containing the mouse TN228 VL and VH variable regions, a human IgG2M3 constant region for the heavy chain, and a human kappa constant region for the light chain. The heavy chain constant region was modified (Cole, M.S., C. Anasetti, and J.Y. Tso. 1997. Human IgG2 variants of chimeric anti-CD3 are nonmitogenic to T cells. J. Immunol. 159: 3613-3621) from the germline human 2 genomic fragment, and the light chain was derived from the germline human K genomic fragment. Both the heavy and light chain genes are driven by the human cytomegalovirus major immediate early promoter and enhancer. The heavy chain gene is followed by the transcription terminator derived from the human complement gene C2 (Ashfield, R., P. Enriquez-Harris, and N.J. Proudfoot. 1991. Transcriptional termination between the closely linked human complement genes C2 and factor B: common termination factor for C2 and c-myc? EMBO J. 10: 4197-4207). The light chain selection marker gpt gene (Mulligan, R.C., and P. Berg. 1981. Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA 78: 2072-2076) and the heavy chain selection marker *dhfr* gene (Simonsen, C.C., and A.D. Levinson. 1983. Isolation and expression of an altered mouse

dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA 80: 2495-2499) are both driven by the SV40 early promoter. For expression of chimeric TN228, transient transfection into COS-7 cells (monkey kidney cell line) was done using lipofectamine (catalog # 10964-013, GIBCO BRL). Spent media from transient transfectants were analyzed for human IgG2M3 antibody production by ELISA, using goat anti-human IgG gamma chain specific antibody as- capturing reagent and HRP-conjugated goat anti-human kappa chain antibody as developing reagent. The spent media was also tested for the ability of ChTN228 to bind to P815/CD28$^+$ cells (stably transfected cell with CD28 into P815 (mouse mastcytoma)) by indirect immunofluorescent staining and analyzed by flow cytometry. For stable cell line -production, the chimeric expression plasmids were transfected into murine myeloma cell line Sp2/0 by electroporation and the transfectants were selected for *gpt* expression. The spent media from stable transfectants were analyzed by ELISA as for the transient transfection.

Results

The cloned V_L and V_H genes were converted into mini-exons by PCR (Fig. 2 and 3) and subcloned into the light and heavy chain expression vectors as described above and shown in Fig. 1.

Transient transfection of COS-7 cells: The chimeric expression vectors were transiently transfected into monkey kidney cell line COS-7 to produce the chimeric TN228$^+$ antibody. Spent medium from the transfected cells was tested by ELISA for the production of chimeric IgG2M3 antibodies and by flow cytometry for binding to P815/CD28$^+$ cells. Spent medium was positive in both assays. The yield of chimeric antibody from transient transfection was ~0.9 μg/ml. The ChTN228 antibody from transient supernatant bound to P815/CD28$^+$ cells in a concentration dependent manner (data not shown).

Stable transfection of Sp2/0 cells: The chimeric expression vectors were transfected into Sp2/0 cells for the production of a stable cell line. Spent media from several transfectants were tested for the production of chimeric TN228 antibody and for binding to P815/CD28$^+$ cells as with the transient transfectants. Most transfectants were positive for both assays. One transfectant was chosen for its higher antibody productivity and expanded to grow in 5 L of serum free medium. ChTN228 was purified from 5 L of spent medium by affinity chromatography. The yield of purified antibody was ~25 mg.

Example 4 Protein purification of chimeric antibody ChTN228

One of the high ChTN228 expressing transfectants from the stable transfection (Clone 7H) was grown in 5 L of GIBCO hybridoma serum-free medium (catalog # 12045-076, GIBCO BRL). Spent culture

supernatant was harvested when cell viability reached 10 % or below, concentrated to 500 ml, and loaded onto a 5 ml protein-A Sepharose column using a Pharmacia P1 pump (2-3 ml/min). The column was washed with PBS before the antibody was eluted with 0.1 M Glycine, 0.1 M NaCl, pH 2.7. The eluted protein was dialyzed against 3 changes of 2 L PBS and then desalted onto a PD-10 column equilibrated with PBS containing an additional 0.1 M NaCl. The desalted protein solution was filtered through a 0.2 µm filter prior to storage at 4 C.

Example 5 Purity determination by size exclusion HPLC and SDS-PAGE

Size exclusion HPLC was performed using a Perkin Elmer HPLC system consisting of a PE ISS 200 Advanced LC Sample Processor, a PE Series 410 Bio LC Pump, a PE 235C Diode Array Detector, and a PE Nelson 600 Series LINK. Perkin Elmer Turbochrom Navigator Version 4.1 software was used to control the autosampler, pump, and detector, and to acquire, store, and process the data. Separation was achieved using two TosoHaas TSK-GEL G3000SWXL size exclusion HPLC columns, 7.8 mm x 300 mm, 5 µm particle size, 250 Å pore size (catalog # 08541, TosoHaas, Montgomeryville, MD) connected in series. The mobile phase was 200 mM potassium phosphate/150 mM potassium chloride at pH 6.9, and the flow rate was 1.00 mL/minute. The column eluate was monitored spectrophotometrically at both 220 nm and 280 nm. The injection volume was 50 µL (50 µg) of the ChTN228 sample.

SDS-PAGE was performed according to standard procedures on a 4-20% gradient gel (catalog # EC6025, Novex, San Diego, CA).

The purity of the isolated ChTN228 was analyzed by size exclusion HPLC and SDS-PAGE. Based on this analysis, the protein is 96.5 % monomer and has the mobility corresponding to a protein of molecular weight ~160 kD. SDS-PAGE analysis of MuTN228, isotype control MuFd79 (mouse IgG1), ChTN228, and isotype control HuEP5C7 (human IgG2M3) under nonreducing conditions also indicated that all four antibodies have a molecular weight of about 150-160 kD. Analysis of the same four proteins under reducing conditions indicated all four antibodies were comprised of a heavy chain with a molecular weight of about 50 kD and a light chain with a molecular weight of about 25 kD.

Example 6. Competition experiment:

Methods

A titration experiment was done using serial two-fold dilutions of MuTN228-FITC antibody beginning at 250 ng/est. P815/CD28$^+$ cells (5x10^5 cells/test) were incubated with FITC-labeled antibody for 1 hour on ice,

washed with PBS and analyzed by flow cytometry. For the competition experiments, 25 ng of MuTN228-FITC and serial two-fold dilutions of competing ChTN228 or MuTN228 antibodies beginning at 800 ng/test were added to P815/CD28$^+$ cells (5x 10^5 cells/test). As a control, P815/CD28$^+$ cells (5x10^5 cells/test) were incubated with 25 ng of MuTN228-FITC alone (i.e. without any competitor). HuEP5C7 and MuFd79 isotype control antibodies (800ng/test) were also tested as competitors. Cells were incubated with the antibody mixture in a final volume of 150 µl for one hour on ice (in the dark), then washed and analyzed by flow cytometry.

Results

The binding specificity of the MuTN228 and ChTN228 antibodies was compared in a flow cytometry competition experiment as described in the Methods. Various amounts of unlabeled MuTN228 or ChTN228 were mixed with 25 ng of FITC-labeled MuTN228 antibody and incubated with P815/CD28' cells. Both MuTN228 and ChTN228 competed with MuTN228-FITC in a concentration dependent manner, indicating that binding of both antibodies is specific for the CD28 antigen (Fig. 4). The isotype control antibodies MuFd79 and HuEP5C7 did not compete with MuTN228-FITC, indicating that the MuTN228 and ChTN228 antibodies recognize the CD28 antigen through V-region specific interactions.

Example 7 Chimeric anti-human CD28 antibody which has reduced affinity to human F R inhibits primary mixed lymphocyte reaction.

Cell preparation

Human peripheral blood mononuclear cells (PBMC) were prepared from normal healthy volunteers by density gradient centrifugation using Ficoll-Paque plus (Amersham Pharmacia Biotech, Tokyo, Japan). Human blood were diluted with equal volume of RPMI1640 and overlaid on Ficoll-Paque plus. After centrifugation for 30 min. at room temperature, PBMCs were collected and washed with RPM11640. Thereafter, PBMCs were suspended with the medium(RPMI1640 containing 2.5% human type AB serum, 2-mercaptoethanol, and antibiotics) and applied to a nylon fiber column(Wako junyaku, Osaka, Japan). After 1 hr incubation at 37 C in 5 % CO_2, T cells were eluted with warm medium.

Human B cell lines (Raji and JY) were used as stimulator cells in the mixed lymphocyte reaction. These cells were X-ray irradiated (2000R) before use.

Primary mixed lymphocyte reaction (1st MLR)

Purified human T cells (1 x 10^5 cells/well) and irradiated Raji (1 x 10^5 cells/well) were plated in 96well flat bottom micro plate. Antibodies were added to the culture medium and cells were incubated for 7 days. All cultures were labeled for final 6 hours with 10 kBq/well [^{3}H]thymidine (Amersham Pharmacia biotech). Cells were harvested and incorporated radioactivity was measured by liquid scintillation counter.

The effect of TN228-IgG2m3 (ChTN228) on primary MLR was shown on Fig.5 and 6. The original anti-human CD28 antibody TN228 (MuTN228) did not inhibit primary MLR, however, chimeric antibody TN228-IgG2m3 inhibited in a dose dependent manner. Therefore, conversion of Fc region of anti-human CD28 antibody to one with reduced affinity to human Fc R makes the antibody antagonistic to T cell proliferation.

Chimeric anti-human CD28 antibody which has reduced affinity to human Fc R reduced T cell low responsiveness in secondary mixed lymphocyte reaction.

Secondary mixed lymphocyte reaction (2nd MLR)

Purified human T cells (1 x 10^5 cells/well) and irradiated Raji cells (1 x 10^5 cells/well) were plated in 96-well flat bottom micro plates. Antibodies were added to the culture medium and cells were incubated. After 5 days, cells were collected, washed with fresh medium. Cells were, suspended with fresh medium and cultured for 8 days. Cells were restimulated with irradiated Raji or JY cells. After additional 7 days culture, cells were incubated with 10 kBq/well [^{3}H]thymidine for 6 hours. Cells were harvested and radioactivity was measured by liquid scintillation counter.

TN228-IgG2m3 inhibited primary MLR (Fig. 5 and 6). Next, we analyzed the effect of this antibody on secondary MLR. The antibody was applied to primary MLR culture, then antibody was removed from culture supernatant. After culturing in the medium without antibodies, cells were re-stimulated with the same stimulator cells(Raji) or third party stimulator(JY). The proliferation of cells treated with TN228-IgG2m3 through primary MLR was reduced compared to that of none-treated cells. However, both cells proliferated to almost the same extent with third party stimulator (JY) (Fig. 7). This result indicates that anti-human CD28 antibody with reduced affinity to human Fc R may induce T-cell energy through alo-antigen stimulation.

Example 8. Design of humanized TN228 variable regions

The V-region sequences of MuTN228 were analyzed by computer modeling. Based on a sequence homology search against the Kabat antibody sequence database (8. Johnson, G., and T.T. Wu. 2000. Kabat database and its applications: 30 years after the first variability plot. Nucleic Acids Res. 28: 214-218), IC4 (Manheimmer-Lory, A., J.B. Katz, M. Pillinger, C. Ghossein, A. Smith, B. Diamond. 1991. Molecular characteristics of antibodies bearing an anti-DNA-associated idiotype. J. Exp. Med. 174: 1639-1652) was selected to provide the framework for both the humanized TN228 heavy chain and light chain variable regions. The humanized TN228 heavy chain variable domain has 65 residues out of 85 framework residues that are identical to those of the mouse TN228 heavy chain framework, or 76% sequence identity. The humanized TN228 light chain variable domain has 56 residues out of 80 framework residues that are identical to those of the mouse TN228 light chain framework, or 70% sequence identity.

The computer programs ABMOD and ENCAD (Levitt, M. 1983. Molecular dynamics of native protein. I. Computer simulations of trajectories. J. Mol. Biol. 168: 595-620) were used to construct a molecular model of the TN228 variable domain, which was used to locate the amino acids in the mouse TN228 framework that are close enough to the CDRs to potentially interact with them. To design the humanized TN228 heavy and light chain variable regions, the CDRs from the mouse TN228 heavy chain were grafted into the framework regions of the human IC4 heavy chain and the CDRs from the mouse TN228 light chain were grafted into the framework regions of the human IC4 light chain. At framework positions where the computer model suggested significant contact with the CDRS, the amino acids from the mouse antibody were substituted for the original human framework amino acids. For humanized TN228, this was done at residues 27, 29, 30, 48, 67, 71 and 78 of the heavy chain. For the light chain, no substitutions were made (i.e., a straight grafting of the MuTN228 CDRs into the IC4 framework region was done). Furthermore, framework residues that occurred only rarely at their positions in the database of human antibodies were replaced by human consensus amino acids at those positions. For humanized TN228 this was done at residues 23, 40, 73, 83 and 85 of the heavy chain and at residues 69 and 77 of the light chain. The amino acid sequences of the humanized TN228 antibody heavy and light chain variable regions are shown Figure 9 and 10.

Example 9. Construction and expression of humanized TN228-IgG2M3

Methods

Once the humanized variable region amino acid sequences had been designed as described above, genes were constructed to encode them, including signal peptides, splice donor signals and appropriate

restriction enzyme sites (Figure 8). The heavy and light chain variable region genes were constructed and amplified using eight overlapping synthetic oligonucleotides ranging in length from approximately 65 to 80 bases (He, X.Y, Z. Xu, J. Melrose, A. Mullowney, M. Vasquez, C. Queen, V. Vexler, C. Klingbeil, M.S. Co, and E.L. Berg. 1998. Humanization and pharmacokinetics of a monoclonal antibody with specificity for both E- and P-selectin. *J. Immunol.* 160: 1029-1035). The oligonucleotides were annealed pairwise and extended with the Klenow fragment of DNA polymerase I, yielding four double-stranded fragments. The resulting fragments were denatured, annealed pairwise, and extended with Klenow, yielding two fragments. These fragments were denatured, annealed pairwise, and extended once again, yielding a full-length gene. The resulting product was amplified by polymerase chain reaction (PCR) using Taq polymerase, gel-purified, digested with XbaI, gel-purified again, and subcloned into the XbaI site of pVg2M3 for the expression of heavy chain, and pVk for the expression of light chain. The pVg2M3 vector for human gamma 2 heavy chain expression (Cole, M.S., C. Anasetti, and J.Y. Tso. 1997. Human IgG2 variants of chimeric anti-CD3 are nonmitogenic to T cells. J. Immunol. 159: 3613-3621), and the pVk vector for human kappa light chain expression (CO, M.S., N.M. Avadalovic, P.C. Caron, M.V. Avadalovic, D.A. Scheinberg, and C. Queen. 1992. Chimeric and humanized antibodies with specificity for the CD33 antigen. J. Immunol. 148:1149-1154) have been previously described.

The sequences of the V-regions and constant region exons of the heavy and light chain final plasmids were verified by nucleotide sequencing. The gross structures of the final plasmids were verified by restriction mapping. All DNA manipulations were performed by standard methods.

In this specification, HuTN228 refers to a humanized antibody containing the humanized TN228 V_H and V_L variable regions, a human IgG2M3 constant region for the heavy chain, and a human kappa constant region for the light chain. The heavy chain constant region was modified (Cole, M.S., C. Anasetti, and J.Y. Tso. 1997. Human IgG2 variants of chimeric anti-CD3 are nonmitogenic to T cells. J. Immunol. 159: 3613-3621) from the germline human 2 genomic fragment, and the light chain was derived from the germline human K genomic fragment. The human cytomegalovirus major immediate early promoter and enhancer drive both the heavy and light chain genes. The heavy chain gene is followed by the transcription terminator derived from the human complement gene C2 (Ashfield, R., P. Enriquez-Harris, and N.J. Proudfoot. 1991. Transcriptional termination between the closely linked human complement genes C2 and factor B: common termination factor for C2 and c-myc? EMBO J. 10: 4197-4207). The light chain selection marker *gpt* gene (Mulligan, R.C., and P. Berg. 1981. Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA 78: 2072-2076) and the heavy chain selection marker

dhfr gene (Simonsen, C.C., and A.D. Levinson. 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA 80: 2495-2499) are both driven by the SV40 early promoter.

For expression of HuTN228, transient transfection into COS-7 cells (monkey kidney cell line) was done using Lipofectamine 2000 (catalog # 11668-027, Life Technologies). Spent media from transient transfectants were analyzed for human IgG2M3 antibody production by ELISA, using goat anti-human IgG gamma chain specific antibody as capturing reagent and HRP-conjugated goat anti-human kappa chain antibody as developing reagent. The spent media were also tested for the ability of HuTN228 to bind to P815/CD28$^+$ cells by indirect immunofluorescent staining and analyzed by flow cytometry (data not shown). For stable cell line production, the humanized expression plasmids were transfected into murine myeloma cell line Sp2/0 by electroporation and the transfectants were selected for gpt expression. The spent media from stable transfectants were analyzed by ELISA as for the transient transfection.

Results

Based on the humanized V-region amino acid sequence design, heavy and light chain V-genes (Figure 9 and 10) were constructed as described in the Methods. The heavy and light chain V-genes were cloned into the pVg2M3 and pVk vectors, respectively, as shown in Figure 8. Several clones were analyzed by nucleotide sequencing and correct clones of both the heavy chain and light chain expression vectors were used for the transfection. The constant regions of both the heavy and light chain expression vectors were also confirmed by sequencing.

Example 10. Expression of HuTN228.

Transient transfection of COS-7 cells: The expression vectors were transiently transfected into monkey kidney cell line COS-7 to produce the HuTN228 antibody. Spent medium from the transfected cells was tested by ELISA for the production of humanized gG2M3 antibodies and by flow cytometry for binding to ^{P815}/CD28$^+$ cells (data not shown). Spent medium was positive in both assays. The yield of humanized antibody from transient transfection was ~3.7 g/ml. The HuTN228 antibody from transient supernatant bound to P815/CD28$^+$ cells in a concentration dependent manner (data not shown).

Stable transfection of Sp2/0 cells: The humanized expression vectors were transfected into Sp2/0 cells for the production of a stable cell line. Spent media from several transfectants were tested for the production of HuTN228 antibody as with the transient transfectants. One transfectant (clone 4) was chosen for its higher antibody productivity and expanded in GIBCO hybridoma serum free medium. HuTN228 antibody was purified from 570 ml of spent medium by affinity chromatography. The yield of purified antibody was ~7 mg.

Example 11. Protein purification.

One of the high HuTN228 expressing transfectants from the stable transfection (Clone 4) was grown in 570 ml of GIBCO hybridoma serum-free medium (catalog # 12045076, Life Technologies). Spent culture supernatant was harvested when cell viability reached 10% or below and loaded onto a 2 ml protein-A Sepharose column. The column was washed with PBS before the antibody was eluted with 0.1 M Glycine, 0.1 M NaCl, pH 2.5. The eluted protein was dialyzed against 3 changes of 2 L PBS and then desalted onto a PD-10 column equilibrated with PBS containing an additional 0.1 M NaCl. The desalted protein solution was filtered through a 0.2 m filter prior to storage at 40C.

Example 11. Purity determination by size exclusion HPLC and SDS-PAGE

Methods

Size exclusion HPLC was performed using a Perkin Elmer HPLC system consisting of a PE ISS 200 Advanced LC Sample Processor, a PE Series 410 Bio LC Pump, a PE 235C Diode Array Detector, and a PE Nelson 600 Series LINK. Perkin Elmer Turbochrolm Navigator Version 4.1 software was used to control the autosampler, pump, and detector, and to acquire, store, and process the data. Separation was achieved using two TosoHaas TSK-GEL G3000SWXL size exclusion HPLC columns (7.8 mm x 300 mm, 5 m particle size, 250 pore size; catalog # 08541, TosoHaas, Montgomeryville, MD) connected in series. The mobile phase was 200 mM potassium phosphate/150 mM potassium chloride at pH 6.9, and the flow rate was 1.00 mL/minute. The column eluate was monitored spectrophotometrically at both 220 nm and 280 nm. The injection volume was 60 l (60 g) of the HuTN228 sample.

SDS-PAGE was performed according to standard procedures on a 4-20% gradient gel (catalog # EC6025, Novex, San Diego, CA).

The isotype of the purified antibody was confirmed using the Human IgG Subclass Profile ELISA Kit (catalog # 99-1000, Zymed Laboratories, South San Francisco, CA) following the manufacturer's recommendations. (Results)

The purity of the isolated HuTN228 antibody was analyzed by size exclusion HPLC and SDS-PAGE. The HPLC elution profile of HuTN228 is not shown. Based on this analysis, the protein is ~98% monomer and has the mobility corresponding to a protein of molecular weight ~160 kD.

SDS-PAGE analysis of MuTN228, isotype control MuFd79 (mouse IgG1), HuTN228, and isotype control HuEP5C7 (human IgG2M3) under nonreducing conditions also indicated that all four antibodies have a molecular weight of about 150-160 kD. Analysis of the same four proteins under reducing conditions indicated that all four antibodies were comprised of a heavy chain with a molecular weight of about 50 kD and a light chain with a molecular weight of about 25 kD.

The isotype test indicated that the isotype of the HuTN228 antibody was consistent with the expected IgG2 isotype (data not shown).

Example 12. FACS competition experiment.

Methods

A titration experiment was done using serial two-fold dilutions of MuTN228-FITC antibody beginning at 250 ng/test. P815/CD28$^+$ cells (3xlO5 cells/test) were incubated with FITC-labeled antibody for 1 hour on ice in 100 l of FACS Staining Buffer (FSB = PBS, 2% FBS, 3% normal mouse serum, 0.1% NaN_3) washed with 2 ml of FSB, and analyzed by flow cytometry (data not shown).

For the competition experiments, MuTN228-FITC (50 ng/test) in 25 l of FSB was combined with three-fold serial dilutions of competing HuTN228 or MuTN228 antibodies (beginning at 200 g/ml) in 25 l of FSB, and added to P815/CD28$^+$ cells ($3x10^5$ cells/test) in 50 l of FSB. As a control, P815/CD28$^+$ cells were incubated with MuTN228-FITC alone (50 ng/test in 50 l of FSB). HuEP5C7 (human IgG2M3) and MuFd79 (mouse IgG1) isotype control antibodies (200 g/ml) in 25 l of FSB were also tested as nonspecific competitors. Cells were incubated with the antibody mixture in a final volume of 100 l for one hour on ice (in the dark), then washed with 2 ml of FSB, and analyzed by flow cytometry. This experiment was repeated three times.

Results

The binding specificity of the MuTN228 and HuTN228 antibodies to CD28 molecules on P815/CD28$^+$ cells was compared in a flow cytometry competition experiment as described in the Methods. A representative result is shown in Figure 5. Both MuTN228 and HuTN228 competed with MuTN228-FITC in a concentration dependent manner, indicating that binding of both antibodies is specific for the CD28 antigen. The relative binding of HuTN228 was a few fold less than that of MuTN228. The isotype control antibodies MuFd79 and

HuEP5C7 did not compete with MuTN228-FITC, indicating that the MuTN228 and HuTN228 antibodies recognize the CD28 antigen through V-region specific interactions.

Example 13. ELISA competition experiment.

Methods

A 96 well ELISA plate (Nunc-Immuno plate, catalog # 439454, NalgeNunc, Naperville, IL) was coated with 100 l/well of sCD28-Fc (0.5 g/ml in PBS) (sCD28-Fc means the fused protein, in which the extracellular domains of CD28 were combined with the CH2 and CH3 domains of IgG1.) overnight at 4 C. The plate was blocked for 30 minutes with 300 l/well of Superblock Blocking Buffer in TBS (catalog # 37535, Pierce, Rockford, IL), and washed with 300 l/well of ELISA Wash Buffer (EWB = PBS, 0.1% Tween-20). A mixture of MuTN228-biotin (0.5 g/ml) in 100 l of ELISA Buffer (EB = PBS, 1% BSA, 0.1% Tween-20) and three-fold serially diluted HuTN228 or MuTN228 competitor antibodies (starting at 100 g/ml) in 100 l of EB was added in triplicate in a final volume of 200 l/well. Isotype control antibodies HuEP5C7 and MuFd79 (100 g/ml) in 100 l of EB were also tested as non-specific competitors. As a 'no competitor' control, 100 l of EB was added to 100 l of MuTN228-biotin (0.5 g/ml). As a blank, 200 l of EB was added to the remaining wells (containing no MuTN228-biotin). The plate was incubated at room temperature for 1.5 hours with shaking. After washing the wells 4 times with 300 l/well of EWB, 100 l/well of Streptavidin-HRP (1 g/ml, catalog # 21124, Pierce) was added to all the wells. The plate was incubated at room temperature for 1 hour with shaking. After washing the wells as above, 100 l/well of ABTS substrate (catalog #507602 & 506502, KPL, Gaithersburg, MD) was added to all the wells. The plate was incubated at room temperature for 5-7 minutes and the optical density was read at 415 nm. This experiment was repeated three times.

Results

The binding specificity of the HuTN228 and MuTN228 antibodies to sCD28-Fc was compared in an ELISA competition experiment as described in the Methods. A representative result is shown in Figure 12. Both MuTN228 and HuTN228 competed with MuTN228-biotin in a concentration dependent manner. The isotype control antibodies MuFd79 and HuEP5C7 did not compete with MuTN228-biotin, indicating that the MuTN228 and HuTN228 antibodies recognize the CD28 antigen through V-region specific interactions. The IC_{50} values of MuTN228 and HuTN228 for all three experiments are shown in Table 2. The relative binding of HuTN228 was on average 2.6 fold less than that of MuTN228.

Table 2. ELISA competition summary

IC_{50} (g/ml)

Competitor	Expt 1	Expt 2	Expt 3	Average	Std. Dev.
MuTN228	0.21	0.20	0.15	0.19	0.03
HuTN228	0.37	0.64	0.48	0.50	0.14

Example 14. ^{125}I-labeled antibody competition experiment

Methods

The relative binding affinities of the MuTN228 and HuTN228 antibodies were determined following the method of Queen et al. (Queen, C., W.P. Schneider, H.E. Selick, P.W. Payne, N.F. Landolfi, J.F. Duncan, N.M. Avdalovic, M. Levitt, R.P. Junghans, T.A. Waldmann. 1989. A humanized antibody that binds to the interleukin 2 receptor. Proc. Natl. Acad. Sci. 86:10029-10033). Briefly, ~10 ng of ^{125}I-labeled MuTN228 in 50 l of Binding Buffer (BB = PBS, 2% FBS, 1 g/ml mouse IgG, 0.1% NaN_3) was combined in triplicate with three-fold serial dilutions of MuTN228 or HuTN228 competitor antibodies (beginning at 400 g /ml) in 50 l of BB, added to 100 l of P815/CD28$^+$ cells (2.5 x 10^5 cells/test) in incubation tubes (Skatron Macrowell Tube Strips, catalog # 15773, Molecular Devices, Sunnyvale, CA), and incubated for 90 minutes at 4 C with gentle shaking. Isotype control antibodies HuEP5C7 and MuFd79 (400 g /ml) in 50 l of BB were also tested as nonspecific competitors. Following the incubation, the cell-antibody mixture was transferred to centrifuge tubes (Sarstedt Micro Tubes, catalog # 72.702, Sarstedt, Newton, NC) containing 0.1 ml 80% dibutyl phthalate-20% olive oil, the incubation tubes were washed once with 50 l of BB, and bound and free counts were separated by centrifugation as described (Kuziel, W.A., S.J. Morgan, T.C. Dawson, S. Griffin, 0. Smithies, K. Ley, N. Maeda. 1997. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. Proc. Natl. Acad. Sci. 94:12053-12058). This experiment was repeated three times.

Results

The relative binding affinities of the MuTN228 and HuTN228 antibodies were compared in an ^{125}I-labeled antibody competition experiment as described in the Method. A representative result is shown in Figure 13. Both MuTN228 and HuTN228 competed with ^{125}I-labeled MuTN228 in a concentration dependent manner. The isotype control antibody MuFd79 showed weak but repeatable competition at high concentrations, but the isotype control antibody HuEP5C7 did not compete with ^{125}I-labeled MuTN228, indicating that the HuTN228 antibody recognizes the CD28 antigen through V-region specific interactions. The IC_{50} values of

MuTN228 and HuTN228 for all three experiments are shown in Table 3. The apparent binding affinity of HuTN228 was approximately 2.4 fold less than that of the MuTN228 antibody.

Table 3. I-125 competition summary

IC_{50} (nM)

Competitor	Expt 1	Expt 2	Expt 3	Average	St. Dev.
MuTN228	0.93	1.05	1.02	1.00	0.06
HuTN228	2.65	2.43	2.13	2.40	0.26

Example 15 Amino acid sequencing of the hamster anti-murine CD28 antibody

Method

Hybridoma and antibodies. The Armenian hamster anti-murine CD28 hybridoma PV1 was obtained from ATCC (ATCC HB-12352). Purified PV1, R-phycoerythrin (R-PE)-conjugated PV1 were purchased from Southern Biotechnology (Birmingham, AL). The Syrian hamster anti-CD28 antibody 37.51 was from PharMingen (San Diego, CA). Secondary antibodies fluorescein (FITC)-conjugated donkey anti-Armenian hamster IgG (H+L), FITC-conjugated donkey anti-Syrian hamster IgG (H+L), FITC-conjugated donkey anti-mouse IgG (H+L), R-PE-F(ab')$_2$ donkey anti-mouse IgG (H+L) were from Jackson ImmunoResearch (West Grove, PA); and FITC-conjugated goat anti-mouse kappa, R-PE-conjugated goat anti-mouse IgG3, and horse radish peroxidase (HRP)-conjugated goat anti-mouse kappa were from Southern Biotechnology. Goat anti-mouse IgG3, and mouse IgG3 isotype control FLOPC 22 were from Sigma Chemicals (St. Louis, MO). The Armenian hamster anti-murine CD3 antibody 145.2C11 and its hamster/mouse chimeric version 145.2C11-IgG3 were generated in our laboratory. FITC-conjugated 145.2C11 was from Boehringer Mannheim (Indianapolis, IN).

Cloning of variable region cDNAs. The V region cDNAs for the light and heavy chains of PV1 were cloned from the hybridoma cells by an anchored polymerase chain reaction (PCR) method described by Co et al. (Co, M.S., N.M. Avadalovic, P.C. Caron, M.V. Avadalovic, D.A. Scheinberg, and C. Queen. 1992. *J. Immunol.* 148:1149-1154.). Amplification was performed on cDNA using 3' primers that anneal respectively to the hamster kappa and gamma chain C regions, and a 5' primer that anneals to the added G-tail of the cDNA. For

V_L PCR, the 3' primer has the sequence of 5'TATAGAGCTCCACTTCCAGTGCCC (SEQ ID NO: 21), with residues 11- 24 hybridizing to the hamster C_k region. For V_H PCR, the 3' primers has the degenerated sequences of (SEQ ID NOS:18, 19 and 20):

```
                                    A          G   T
5'TATAGAGCTCAAGCTTCCAGTGGATAGACCGATGGGGCTGTCGTTTTGGC,
                                    T
```

with residues 19 - 50 hybridizing to most rodent IgG C_H1. The non-hybridizing sequences in the two primer sets contain restriction sites used for cloning. The V_L and V_H cDNAs were subcloned into a pUC19 vector for sequence determination. To avoid PCR-generated errors, five independent clones for each cDNA were sequenced, and only the clones whose sequence agreed with the consensus sequence were chosen to express the chimeric PV1.

Results

Cloning of PV1 V region cDNAs. The PV1 light and heavy chain V region cDNAs were cloned from the hybridoma cells as described in Methods. For the V_L PCR, only 3' primer corresponding to the hamster C_γ region could yield V_L cDNA product from PV1. A 3' primer from the hamster C_γ region, on the other hand, did not yield any PCR product. These results indicated that the hybridoma PV1 uses kappa for its light chain. Several light and heavy chain clones were sequenced and were found to contain the same V_L and V_H, respectively. Limited C_H1 and C_γ sequence data indicated that the cloned heavy and light chains are not murine in origin.

Example 16 Construction and expression of chimeric PV1-IgG3.

Method

PV1 V_L and V_H were made by PCR into mini-exon segments flanked by XbaI sites as described (He, X.Y., Z. Xu, J. Melrose, A. Mullowney, M. Vasquez, C. Queen, V. Vexler, C. Klingbeil, M.S. Co, and E.L. Berg. 1998. *J. Immunol.* 160:1029-1035.) and they were separately introduced to the light chain and heavy chain expression plasmids (Fig. 14). Each mini-exon contains a signal peptide sequence, a mature variable region sequence and a 5' splicing donor sequence derived from the most homologous mouse J chain gene. Such splicing donor is used to splice the V region exon to the mouse antibody constant region. Each mini-exon was sequenced again after it

had been cloned into the expression vector to ensure the correct splicing signal was introduced, and no PCR errors were generated.

A vector was constructed to express both the heavy and light chain genes of the chimeric PV1-IgG3 from a single plasmid. In this report, PV1-IgG3 refers to a chimeric antibody containing the hamster PV1 V_L and V_H variable regions, a mouse IgG3 constant region for the heavy chain, and a mouse kappa constant region for the light chain. The expression vector pV1.g3.rg.dE (Fig. 14) was obtained by a two-step cloning process similar to that described by Cole et. al. (Cole, M.S., C. Anasetti, and J.Y. Tso. 1997. *J. Immunol.* 159:3613-3621.). The heavy chain constant region was derived from the mouse γ3 genomic fragment, and the light chain from the κ fragment. Both the heavy and light chain genes are driven by the human cytomegalovirus major immediate early promoter and enhancer, and they are separated by the transcription terminator derived from the human complement gene C2 (Ashfield, R., P. Enriquez-Harris, and N.J. Proudfoot. 1991. *EMBO J.* 10:4197-4207.). The selection marker *gpt* gene (Mulligan, R.C., and P. Berg. 1981. *Proc. Natl. Acad. Sci. USA* 78:2072-2076) is driven by a modified SV40 early promoter. For expression of the chimeric PV1-IgG3, the single plasmid vector was transfected into the murine myeloma cell line NS0, and the transfectants were selected for *gpt* expression. Spent media from transfectants were analyzed for mouse IgG3 antibody production by ELISA, using goat anti-mouse IgG3 as capturing reagent and HRP-conjugated goat anti-mouse kappa chain as developing reagent. The assay is specific for mouse IgG3; other mouse IgG isotypes are negative in this analysis.

Results

Expression of the chimeric PV1-IgG3. The cloned V_L and V_H were made into mini-exons (Fig. 15) and incorporated into an expression vector as described in *Materials and Methods* and Fig. 15. The expression vector was then transfected into a murine myeloma cell line NS0 to produce the chimeric PV1-IgG3. Spent media from several transfectants were assayed by ELISA for the production of mouse IgG3 antibodies and by FACScan for binding to *EL4* cells. Most transfectants were positive in both assays. One transfectant was chosen for its high antibody productivity and expanded to grow in 1 L of serum-free medium. PV1-IgG3 was purified from the 1 L spent medium by affinity chromatography. The yield was >10 mg/L.

Example 17 Characterization of the purified chimeric PVI-IgG3 by HPLC and SDS-PAGE

Methods

Protein Purification. One of the high IgG3-expressing transfectants (Clone #1) was grown in 1 L of Gibco Serum-free Hybridoma medium. Spent culture supernatant was harvested when cell viability reached 30 % or below, concentrated to 200 ml, and loaded onto a 5 ml protein-A Sepharose column using a Pharmacia P1 pump (2-3 ml/min). The column was then washed with PBS containing an additional 0.1 M NaCl (final concentration of NaCl was 0.25 M) before the antibody was eluted with 3.5 M $MgCl_2$. The eluted protein was then desalted onto a PD10 column equilibrated with PBS containing an additional 0.1 M NaCl. The desalted protein solution was filtered through a 0.2 μm filter prior to storage at 4 °C. Like all mouse IgG3, PV1-IgG3 at high concentrations (>1 mg/mL) precipitates in the cold but returns to solution by warming at 37° C. The antibody stays in solution at room temperature. Repeated cycles of cold precipitation do not seem to affect the antigen binding activity of the antibody.

Purity Determination by size exclusion HPLC and SDS-PAGE. Size exclusion HPLC was performed using a Perkin Elmer HPLC system consisting of a PE ISS 200 Advanced LC Sample Processor, a PE Series 410 Bio LC Pump, a PE 235C Diode Array Detector, and a PE Nelson 600 Series LINK. Perkin Elmer Turbochrom Navigator Version 4.1 software was used to control the autosampler, pump, and detector, and to acquire, store, and process the data. Separation was achieved using two TosoHaas TSK-GEL G3000SWXL size exclusion HPLC columns (TosoHaas, catalog # 08541, 7.8 mm x 300 mm, 5 μm particle size, 250 Å pore size) connected in series. The mobile phase was 200 mM potassium phosphate/150 mM potassium chloride at pH 6.9, and the flow rate was 1.00 mL/minute. The column eluate was monitored spectrophotometrically at both 220 nm and 280 nm. The injection volume was 50 μL (63.5 μg) of the undiluted PV1-IgG3 sample. SDS-PAGE was performed according to standard procedures.

Results

. The purity of the isolated PV1-IgG3 was analyzed by size exclusion HPLC and SDS-PAGE. The HPLC elution profile of PV1-IgG3 is shown in Fig. 16. Based on this analysis, the protein is 99% monomer and has the mobility corresponding to the molecular weight of 150 kD. SDS-PAGE analysis of PV1, PV1-IgG3 and isotype control under nonreducing conditions also indicated all three antibodies have the molecular weight of

about 150 kD (Fig. 17A). The minor bands seen in Fig. 17A were artifacts due to boiling of the samples in SDS without reduction. They reflected the number of incomplete inter-chain disulfide bonds in the antibodies. Analysis of the same three proteins under reducing conditions (Fig. 17B), however, indicated that PV1, but not PV1-IgG3 or the isotype control, has a heavy chain with molecular weight slightly higher than the 50 kD molecular weight usually seen with IgG. The hamster antibody PV1 thus either has heavy glycosylation at Asn_{297} in C_H3, or it has an extra glycosylation site elsewhere in the heavy chain. As discussed later, this unusual glycosylation pattern may contribute to PV1's nonspecific binding to EL4 cells, perhaps by lectin/carbohydrate interaction.

Example 18

Methods

Flow cytometry. Murine T cell line EL4 cells (2.5×10^5 cells/0.2 ml) were stained with 1 μg/ml of PV1, 37.51 or PVI-IgG3 at 4° C for 30 min, washed with 2 ml of cold PBS, and stained with 20 μl of specific fluorochrome-conjugated secondary antibodies (10 μg/ml). After 20 min of incubation at 4° C in the dark, the cells were washed with PBS and analyzed by FACScan (Becton Dickenson, Milpitas, CA).

In the competition experiment, EL4 cells (2.5×10^5 cells/0.2 ml) were stained with 1 μg/ml of R-PE-PV1 and 25 μg/ml of PV1, PV1-IgG3, or IgG3 isotype control at 4° C for 30 min in the dark, washed with PBS and analyzed by FACScan. Similar competition experiment was also conducted using various versions of 145.2C11. In the reverse competition experiment, EL4 cells (2.5×10^5 cells/0.2 ml) were stained with 1 μg/ml of PV1-IgG3 and 25 μg/ml of PV1 at 4° C for 30 min, washed 2 times with PBS, stained with FITC-conjugated donkey anti-mouse IgG (H+L), washed, and analyzed by FACScan. To control for nonspecific binding of the secondary antibodies to PV1, EL4 cells were stained with excess PV1 without PV1-IgG3 and analyzed.

For mouse T cell staining, BALB/c mouse splenic cells (2.5×10^5 cells/0.2 ml) were stained with 1 μg/ml of mouse IgG3 isotype control (FLOPC 21) or PVI-IgG3 at 4° C for 30 min, washed with 2 ml of cold PBS, and stained with 20 μl of FITC-conjugated 145.2C11 (10 μg/ml) and 20 μl of R-PE-conjugated goat anti-mouse

IgG3 (10 μg/ml). After 20 min of incubation at 4° C in the dark, the cells were washed with PBS and analyzed by FACScan.

Results

Characterization of PV1 and PV1-IgG3 by flow cytometry. PV1 was used to stain CD28-positive T cell line EL4 and analyzed by FACScan. The pattern of staining indicated that PV1 binds EL4 cells at two different sites (Fig. 17A). In addition, PV1 as well as several Armenian hamster anti-murine T cell antibodies (145.2C11, anti-CD3; H57-597, anti-TCR; and UC10-4F10-11, anti-CTLA4) also bind nonspecifically to CD28-negative myeloma cell line NS0 (data not shown). The Syrian hamster anti-CD28 antibody 37.51, on the other hand, binds specifically to only one site on EL4 cells (Fig. 17B). It appears that, in addition to CD28 binding, PV1 also binds nonspecifically to other sites, possibly through the carbohydrate/lectin type of interaction. As shown in Fig. 17C, the chimeric PV1-IgG3 does not contain this nonspecific binding activity. The antibody binds EL4 cells in a pattern similar to that of 37.51, and it does not bind to CD28-negative NS0 cells (data not shown). Thus, the nonspecific binding property of PV1 lies in the heavy chain constant region of this particular antibody and it is eliminated upon chimerization.

To demonstrate that PV1-IgG3 contains the CD28-specific binding activity, we used the FACScan competition assay. In these experiments, R-PE-conjugated PV1 was mixed with excess (25-fold) unlabeled PV1, PV1-IgG3 or mouse IgG3 control, and the mixture was used to stain EL4 cells. As shown in Fig. 18A, both PV1 and PV1-IgG3, but not isotype control, prevented R-PE-conjugated PV1 from binding to EL4 cells. The inhibition by PV1-IgG3 was less than that by PV1, and we interpreted these data as PV1-IgG3 competed with R-PE-conjugated PV1 for the CD28 sites but not for the nonspecific sites. Similarly, both 145.2C11(Armenian hamster anti-murine CD3) and the chimeric 145.2C11-IgG3 prevented R-PE-conjugated 145.2C11 from binding to EL4 cells (Fig. 18B), but the chimeric antibody is less efficient due to its inability to eliminate R-PE-145.2C11's nonspecific binding to cells.

We also did the reverse competition experiment using excess (25-fold) PV1 to compete with PV1-IgG3 for binding to EL4 cells. Although PV-1-IgG3 was not labeled in this case, it was specifically recognized by the FITC-conjugated donkey anti-mouse antibodies. The results in Fig. 18C showed that the inhibition of PV1-

IgG3's binding to EL4 cells by excess PV1 was almost complete, demonstrating that PV1 and PV1-IgG3 bind to the same epitope.

Finally, PV1-IgG3 was used to stain mouse splenic cells. PV1-IgG3-coated splenic cells were specifically recognized by the secondary antibodies R-PE-conjugated goat anti-mouse IgG3. Simultaneously, FITC-conjugated 145.2C11 was also added to splenic cells to label CD3-positive cells. In the two-color flow cytometry analysis. PV1-IgG3 specifically stained CD3-positive cells, but not CD3-negative cells (Fig. 19B). Mouse IgG3 isotype control, on the other hand, did not stain the CD3-positive cells (Fig. 19A). Thus, the chimeric PV1-IgG3 recognizes an antigen that is expressed on murine T cells, an antigen binding activity that is consistent with an anti-CD28 antibody.

Example 19 Induction of Collagen Induced Arthritis

Methods

Mice were immunized intradermally at the base of the tail with 125 μg of bovine CII (Collagen Gijutsu Kenkyukai, Japan) emulsified with an equal volume of CFA (Wako, Japan). Mice were boosted by intradermal injection with 125μg of bovine CII in CFA on day 21. Mice were treated anti-CD28 antibody (PV1-IgG3) at the dose of 1mg/kg/day continuous infusion via osmotic pump for 7days after the initial immunization. Arthritis development was checked by inspection of four paws on day 11 after the second immunization, and the inflammation of four paws was graded from 0 to 3 as described previously (Tada, Y., A. Ho, D.-R. Koh, T. W. Mak. 1996. J. Immunol. 156:4520, . Tada, Y., A. Ho, T. Matsuyama, T. W. Mak. 1997. J. Exp. Med. 185:231). Each paw was graded and the four scores were added such that the maximal score per mouse was 12. The arthritis index was calculated by dividing the total score of the experimental mice by the number of the total number of mice.

Results

Mice were immunized with bovine CII, and observed for development of arthritis. At day 11 after the second immunization, arthritis index was significantly reduced in mice treated with anti-CD28 antibody (0.63±0.50) ($P<0.01$) versus control (7.50±0.66).

Example 20

Methods

Mice;Animals

Female BALB/c and C3H mice were obtained from Charles River Japan, Inc. (Yokohama, Japan). Animals were all housed in a specific pathogen-free facility in microisolator cages with filtered air and free access to food and water. All mice were 6–8 wk of age when experiments were initiated.

Antibodies;

Anti-mouse silent CD28 (PV1-IgG3) has identical specificity to that of PV-1 clone but it does not have strong agonistic activity in vitro (Fc→IgG3). Anti-mouse CD154 (TRAP1, IgG1) was purchased from BD PharMingen (San Diego, CA). CTLA4-Ig (CTLA-4/Fc Chimera) was purchased from Genzyme (Cambridge, MA).

Tail-Skin transplantation;

Full thickness skin grafts (0.5 cm2) from tail of donor mice(BALB/c:H-2d) were transplanted on the dorsal thorax of recipient mice(C3H:H-2b) and secured with a band-aid for 7 days. Graft survival was then followed by daily visual inspection. Rejection was defined as the >80% loss of viable epidermal graft tissue. Statistical analyses were performed using a Dunnett's Multiple Comparison test. Values of $p < 0.05$ were considered significant.

Treatment protocols;

Skin graft recipients were treated with 10, 50, 250 μg of anti-mouse silent CD28,

250 μg of anti-mouse CD154 and 100 μg of CTLA4-Ig administered i.p. on the day of transplantation (day 0) and on postoperative days 3, and 6.

Results;

Simultaneous blockade of the CD40 and CD28 T cell costimulatory pathways by administration of anti-mouse silent CD28 and anti-mouse CD154 effectively promotes skin allograft survival in C3H mice. Control animals rejected their grafts at 9 days. Anti-CD40L mAb alone modestly prolonged allograft survival (MST 10 days), but was seen to dramatically improve survival when combined with CD28 extending median survival time-(MST-) beyond 33 days. This strategy is markedly less effective in administration of CTLA4-Ig and anti-mouse CD40L mAb, with MST of 12 days.

Example 21 Preparation of Fab and F(ab')2 fragment and of anti-CD28 antibody

Preparation of Fab fragment of anti-CD28 antibody

Anti-human CD28 antibody (HuTN228) was digested with immobilized-Ficin (Pierce, USA). Immobilized ficin was activated with 50mM Tris-HCL pH 6.8 buffer containing 5mM EDTA and 11.5mM cysteine·HCl and packed to a column. Antibody solution was added to the column, and incubated at 37℃ for 2 or 3 days. The column was washed with PBS and the digest was concentrated by ultrafiltration. The concentrated digest was applied to the gel-filtration column (TSKgel-3000SWxl, Tosoh, Japan) and appropriate fractions were collected and concentrated by ultrafiltration. Protein concentration was determined by absorbance at 280nm (Abs280 = 1.4 for 1mg/mL) and the fragment size was confirmed by SDS-PAGE.

Preparation of F(ab')2 fragment of anti-CD28 antibody

Anti-human CD28 antibody was prepared with the same method as that of Fab fragment except for the concentration of cysteine (1.15mM) and the period of incubation (one over night).

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

What is claimed is:

1. A silenced anti-CD28 antibody.

2. The antibody of Claim 1 which is a chimeric antibody.

3. The antibody of Claim 1 which is a humanized antibody.

4. The antibody of Claim 1 which has a variable region comprising the amino acid sequence in SEQ ID NO:2 or SEQ ID NO:4.

5. The antibody of Claim 1 which has a variable region comprising the amino acid sequence in SEQ ID NO:2 and SEQ ID NO:4.

6. The antibody of Claim 1 which has a variable region comprising the amino acid sequence in SEQ ID NO:7 or SEQ ID NO:9.

7. The antibody of Claim 1 which has a variable region comprising the amino acid sequence in SEQ ID NO:7 and SEQ ID NO:9.

8. A polynucleotide encoding the antibody of Claim 1.

9. The polynucleotide of Claim 8 which comprises at least one polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:6, and SEQ ID NO:8.

10. An expression vector comprising the polynucleotide of Claim 8.

11. A host cell comprising the polynucleotide of Claim 8.

12. A host cell comprising the expression vector of Claim 10.

13. A method of producing a silenced anti-CD28 antibody comprising

culturing the host cell of Claim 11 under conditions suitable for expression of the antibody and recovering the expressed antibody from said culture.

14. A method of producing a silenced anti-CD28 antibody comprising

culturing the host cell of Claim 12 under conditions suitable for expression of the antibody and recovering the expressed antibody from said culture.

15. A method of producing a silenced anti-CD28 antibody comprising

introducing the polynucleotide of Claim 8 into a host cell;

culturing the host cell under conditions suitable for expression of the antibody; and recovering the expressed antibody from said culture.

16. The method of Claim 15 wherein said polynucleotide comprises at least one polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:6, and SEQ ID NO:8.

17. A method of producing a silenced anti-CD28 antibody comprising

introducing the expression vector of Claim 10 into a host cell;

culturing the host cell under conditions suitable for expression of the antibody; and recovering the expressed antibody from said culture.

18. A pharmaceutical composition comprising the silenced anti-CD28 antibody of Claim 1 and a pharmaceutically acceptable ingredient.

19. Use of an effective amount of the antibody of Claim 1 for the manufacture of a medicament to induce T-cell tolerance in a patient.

20. The use of Claim 19, wherein said medicament further comprises another immunosuppressant.

21. Use of an effective amount of the antibody of Claim 1 for the manufacture of a medicament to provide immunosuppression in a patient.

22. The use of Claim 21, wherein said medicament further comprises another immunosuppressant.

23. Use of an effective amount of the antibody of Claim 1 for the manufacture of a medicament to treat organ or tissue transplant rejection in a patient.

24. The use of Claim 23, wherein said medicament further comprises another immunosuppressant.

25. An antibody HuTN228.

SEQUENCE LISTING

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TAMURA, Kouichi
HIGASHI, Yauyuki
SEKI, Nobuo
UEDA, Hirotsugu
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Pro Ser Cys Val Leu Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly
    15                  20                  25

ctg gtg aag ccc tca gag acc ctg tcc ctc act tgc gct gtc tct gga     146
Leu Val Lys Pro Ser Glu Thr Leu Ser Leu Thr Cys Ala Val Ser Gly
30                  35                  40                  45

ttt tca tta acc agc tat ggt gta cac tgg att cgc cag cct cca gga     194
Phe Ser Leu Thr Ser Tyr Gly Val His Trp Ile Arg Gln Pro Pro Gly
                50                  55                  60

aag ggt ctg gaa tgg ctg gga gtc ata tgg cct ggt gga ggc aca aat     242
Lys Gly Leu Glu Trp Leu Gly Val Ile Trp Pro Gly Gly Gly Thr Asn
            65                  70                  75

ttt aat tcg gct ctc atg tcc aga ctg acc atc agc gaa gac acc tcc     290
Phe Asn Ser Ala Leu Met Ser Arg Leu Thr Ile Ser Glu Asp Thr Ser
        80                  85                  90

aag aac caa gtt tcc tta aaa ttg agc tct gtg aca gct gct gac aca     338
Lys Asn Gln Val Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr
    95                  100                 105

gcc gta tat tat tgt gcc aga gat cgg gcg tat ggt aac tac ctc tat     386
Ala Val Tyr Tyr Cys Ala Arg Asp Arg Ala Tyr Gly Asn Tyr Leu Tyr
110                 115                 120                 125

gcg atg gac tac tgg ggt caa gga acc tta gtc acc gtc tcc tca         431
Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                130                 135                 140

ggtaagaatg gcctctaga                                               450


<210>  7
<211>  140
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<212> PRT
<213> hybrid

<400> 7

Met Ala Val Leu Val Leu Phe Leu Cys Leu Val Ala Phe Pro Ser Cys
1 5 10 15

Val Leu Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys
20 25 30

Pro Ser Glu Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Phe Ser Leu
35 40 45

Thr Ser Tyr Gly Val His Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu
50 55 60

Glu Trp Leu Gly Val Ile Trp Pro Gly Gly Gly Thr Asn Phe Asn Ser
65 70 75 80

Ala Leu Met Ser Arg Leu Thr Ile Ser Glu Asp Thr Ser Lys Asn Gln
85 90 95

Val Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr
100 105 110

Tyr Cys Ala Arg Asp Arg Ala Tyr Gly Asn Tyr Leu Tyr Ala Met Asp
115 120 125

Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
130 135 140

<210> 8
<211> 427
<212> DNA
<213> hybrid

<220>
<221> CDS
<222> (12)..(404)
<223>

<400> 8
tctagaccac c atg gag tca gac aca ctc ctg cta tgg gtg ctg ctg ctc 50
Met Glu Ser Asp Thr Leu Leu Leu Trp Val Leu Leu Leu
1 5 10

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tgg gtt cca ggc tcc act ggt gac att cag atg acc caa tct cca tct        98
Trp Val Pro Gly Ser Thr Gly Asp Ile Gln Met Thr Gln Ser Pro Ser
    15                  20                  25

tct ttg tct gcg tct gtg ggg gac agg gtc acc atc aca tgc aga gcc       146
Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala
30                  35                  40                  45

agt gaa agt gtt gaa tat tat gtc aca agt tta atg cag tgg tac caa       194
Ser Glu Ser Val Glu Tyr Tyr Val Thr Ser Leu Met Gln Trp Tyr Gln
                50                  55                  60

cag aaa cca gga aag gca ccc aaa ctc ctc atc tat gct gca tcc aac       242
Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ala Ala Ser Asn
            65                  70                  75

gta gat tct ggg gtc cct tcc agg ttt agt ggc agt ggg tct ggg aca       290
Val Asp Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr
        80                  85                  90

gac ttc acc ctc acc atc tct tct ctg cag ccg gag gat att gca acg       338
Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr
    95                  100                 105

tat tac tgt cag caa agt agg aag gtt cca ttc acg ttc ggc ggg ggg       386
Tyr Tyr Cys Gln Gln Ser Arg Lys Val Pro Phe Thr Phe Gly Gly Gly
110                 115                 120                 125

aca aag gtg gaa ata aaa cgtaagtaga cttttgctct aga                     427
Thr Lys Val Glu Ile Lys
                130
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<210> 9
<211> 131
<212> PRT
<213> hybrid

<400> 9

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Met Glu Ser Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1               5                   10                  15

Gly Ser Thr Gly Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
            20                  25                  30

Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Glu Ser
        35                  40                  45

Val Glu Tyr Tyr Val Thr Ser Leu Met Gln Trp Tyr Gln Gln Lys Pro
    50                  55                  60

Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ala Ala Ser Asn Val Asp Ser
```

65 70 75 80

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
85 90 95

Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys
100 105 110

Gln Gln Ser Arg Lys Val Pro Phe Thr Phe Gly Gly Gly Thr Lys Val
115 120 125

Glu Ile Lys
130

<210> 10
<211> 445
<212> DNA
<213> hybrid

<220>
<221> CDS
<222> (21)..(419)
<223>

<400> 10
tctagacagt ggggaacaat atg gat tca cag atc cag gtc ctc atg tcc ctg 53
Met Asp Ser Gln Ile Gln Val Leu Met Ser Leu
1 5 10

ctc ctc tgg atg tct ggt gcc tgt gga gat att gtg atg acc cag tct 101
Leu Leu Trp Met Ser Gly Ala Cys Gly Asp Ile Val Met Thr Gln Ser
15 20 25

cca tat tcc ctg gct gtg tca gca gga gag aag gtc acc atg agt tgc 149
Pro Tyr Ser Leu Ala Val Ser Ala Gly Glu Lys Val Thr Met Ser Cys
30 35 40

agg tcc agt cag agc ctc tat tac agt gga atc aaa aag aac ctc ttg 197
Arg Ser Ser Gln Ser Leu Tyr Tyr Ser Gly Ile Lys Lys Asn Leu Leu
45 50 55

gcc tgg tac cag cag aaa cca ggc cag tct ccg aaa ctg ctg atc tac 245
Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr
60 65 70 75

ttt aca tct act cgg tta cct ggg gta ccg gat cgc ttc aca ggc agt 293
Phe Thr Ser Thr Arg Leu Pro Gly Val Pro Asp Arg Phe Thr Gly Ser
80 85 90

gga tct ggg aca gat tac act ctc acc atc acc agt gtc cag gct gaa 341
Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Thr Ser Val Gln Ala Glu

```
            95                  100                 105

gac atg ggg cat tat ttc tgt cag cag ggt ata agc act ccg ctc acg      389
Asp Met Gly His Tyr Phe Cys Gln Gln Gly Ile Ser Thr Pro Leu Thr
        110                 115                 120

ttc ggt gat ggc acc aag ctg gag ata aga cgtaagtaga atccaaagtc      439
Phe Gly Asp Gly Thr Lys Leu Glu Ile Arg
    125                 130

tctaga                                                            445
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<210> 11
<211> 133
<212> PRT
<213> hybrid

<400> 11

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Met Asp Ser Gln Ile Gln Val Leu Met Ser Leu Leu Leu Trp Met Ser
1               5                   10                  15

Gly Ala Cys Gly Asp Ile Val Met Thr Gln Ser Pro Tyr Ser Leu Ala
            20                  25                  30

Val Ser Ala Gly Glu Lys Val Thr Met Ser Cys Arg Ser Ser Gln Ser
        35                  40                  45

Leu Tyr Tyr Ser Gly Ile Lys Lys Asn Leu Leu Ala Trp Tyr Gln Gln
    50                  55                  60

Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Phe Thr Ser Thr Arg
65                  70                  75                  80

Leu Pro Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp
                85                  90                  95

Tyr Thr Leu Thr Ile Thr Ser Val Gln Ala Glu Asp Met Gly His Tyr
            100                 105                 110

Phe Cys Gln Gln Gly Ile Ser Thr Pro Leu Thr Phe Gly Asp Gly Thr
        115                 120                 125

Lys Leu Glu Ile Arg
    130
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<210> 12

<211> 467
<212> DNA
<213> hybrid

<220>
<221> CDS
<222> (16)..(444)
<223>

<400> 12
tctagagtct tcacc atg gta tgg ggc ttg atc atc atc ttc ctg gtc aca 51
Met Val Trp Gly Leu Ile Ile Ile Phe Leu Val Thr
1 5 10

gca gct aca ggt gtc cac tcc cag gtc cag ttg aag cag tct ggg gct 99
Ala Ala Thr Gly Val His Ser Gln Val Gln Leu Lys Gln Ser Gly Ala
15 20 25

gag ctt gtg aag cct gga gcc tca gtg aag ata tcc tgc aaa act tca 147
Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Thr Ser
30 35 40

ggc tat acc ttc act gat ggc tac atg aac tgg gtt gag cag aag cct 195
Gly Tyr Thr Phe Thr Asp Gly Tyr Met Asn Trp Val Glu Gln Lys Pro
45 50 55 60

ggg cag ggc ctt gag tgg att gga aga att gat cct gat agt ggt aat 243
Gly Gln Gly Leu Glu Trp Ile Gly Arg Ile Asp Pro Asp Ser Gly Asn
65 70 75

act cgg tac aat cag aaa ttc cag ggc aag gcc aca ctg act aga gac 291
Thr Arg Tyr Asn Gln Lys Phe Gln Gly Lys Ala Thr Leu Thr Arg Asp
80 85 90

aaa tcc tcc agc aca gtc tac atg gac ctc agg agc ctg aca tct gag 339
Lys Ser Ser Ser Thr Val Tyr Met Asp Leu Arg Ser Leu Thr Ser Glu
95 100 105

gac tct gct gtc tat tac tgt gcg aga gat ggg acc ttc tac ggt acc 387
Asp Ser Ala Val Tyr Tyr Cys Ala Arg Asp Gly Thr Phe Tyr Gly Thr
110 115 120

tac ggc tac tgg tac ttc gat ttc tgg ggc cag ggg acc cag gtc acc 435
Tyr Gly Tyr Trp Tyr Phe Asp Phe Trp Gly Gln Gly Thr Gln Val Thr
125 130 135 140

gtc tcc tca ggtgagtcct taaaacctct aga 467
Val Ser Ser

<210> 13
<211> 143
<212> PRT
<213> hybrid

<400> 13

Met Val Trp Gly Leu Ile Ile Ile Phe Leu Val Thr Ala Ala Thr Gly
1 5 10 15

Val His Ser Gln Val Gln Leu Lys Gln Ser Gly Ala Glu Leu Val Lys
20 25 30

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Gly Tyr Met Asn Trp Val Glu Gln Lys Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Arg Ile Asp Pro Asp Ser Gly Asn Thr Arg Tyr Asn
65 70 75 80

Gln Lys Phe Gln Gly Lys Ala Thr Leu Thr Arg Asp Lys Ser Ser Ser
85 90 95

Thr Val Tyr Met Asp Leu Arg Ser Leu Thr Ser Glu Asp Ser Ala Val
100 105 110

Tyr Tyr Cys Ala Arg Asp Gly Thr Phe Tyr Gly Thr Tyr Gly Tyr Trp
115 120 125

Tyr Phe Asp Phe Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
130 135 140

<210> 14
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic DNA

<400> 14
tatagagctc aagcttggat ggtgggaaga tggatacagt tggtgc 46

<210> 15
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic DNA

<400> 15
tatagagctc aagcttccag tggatagacc gatggggctg tcgttttggc 50

<210> 16
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic DNA

<400> 16
tatagagctc aagcttccag tggatagaca gatgggggtg ttgttttggc 50

<210> 17
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic DNA

<400> 17
tatagagctc aagcttccag tggatagacc gttggggctg tcgttttggc 50

<210> 18
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic DNA

<400> 18
tatagagctc aagcttccag tggatagacc gatggggctg tcgttttggc 50

<210> 19
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic DNA

<400> 19
tatagagctc aagcttccag tggatagacc gatgggggtg ttgttttggc 50

<210> 20
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic DNA

<400> 20
tatagagctc aagcttccag tggatagtcc gatggggctg tcgttttggc 50

<210> 21
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic DNA

<400> 21
tatagagctc cacttccagt gccc 24