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(54) **KNOBS AND HOLES HETEROMERIC POLYPEPTIDES**

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(63) Continuation of application No. 11/533,709, filed on Sep. 20, 2006, now Pat. No. 7,695,936, which is a continuation of application No. 10/010,245, filed on Dec. 7, 2001, now Pat. No. 7,642,228, which is a continuation of application No. 08/974,183, filed on Nov. 19, 1997, now abandoned, which is a continuation of application No. 08/399,106, filed on Mar. 1, 1995, now Pat. No. 5,731,168.

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**C07K 19/00** (2006.01)  
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(52) **U.S. Cl.** ..... **435/69.1**; 435/69.7; 435/70.1; 435/71.1; 435/325; 530/350; 530/387.1; 530/387.3; 530/300; 424/130.1; 424/133.1; 424/136.1; 424/178.1

(58) **Field of Classification Search** ..... None  
See application file for complete search history.

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(57) **ABSTRACT**

The invention relates to a method of preparing heteromultimeric polypeptides such as bispecific antibodies, bispecific immuno adhesins and antibody-immuno adhesin chimeras. The invention also relates to the heteromultimers prepared using the method. Generally, the method involves introducing a protuberance at the interface of a first polypeptide and a corresponding cavity in the interface of a second polypeptide, such that the protuberance can be positioned in the cavity so as to promote heteromultimer formation and hinder homomultimer formation. "Protuberances" are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). The protuberance and cavity can be made by synthetic means such as altering the nucleic acid encoding the polypeptides or by peptide synthesis.

**36 Claims, 11 Drawing Sheets**

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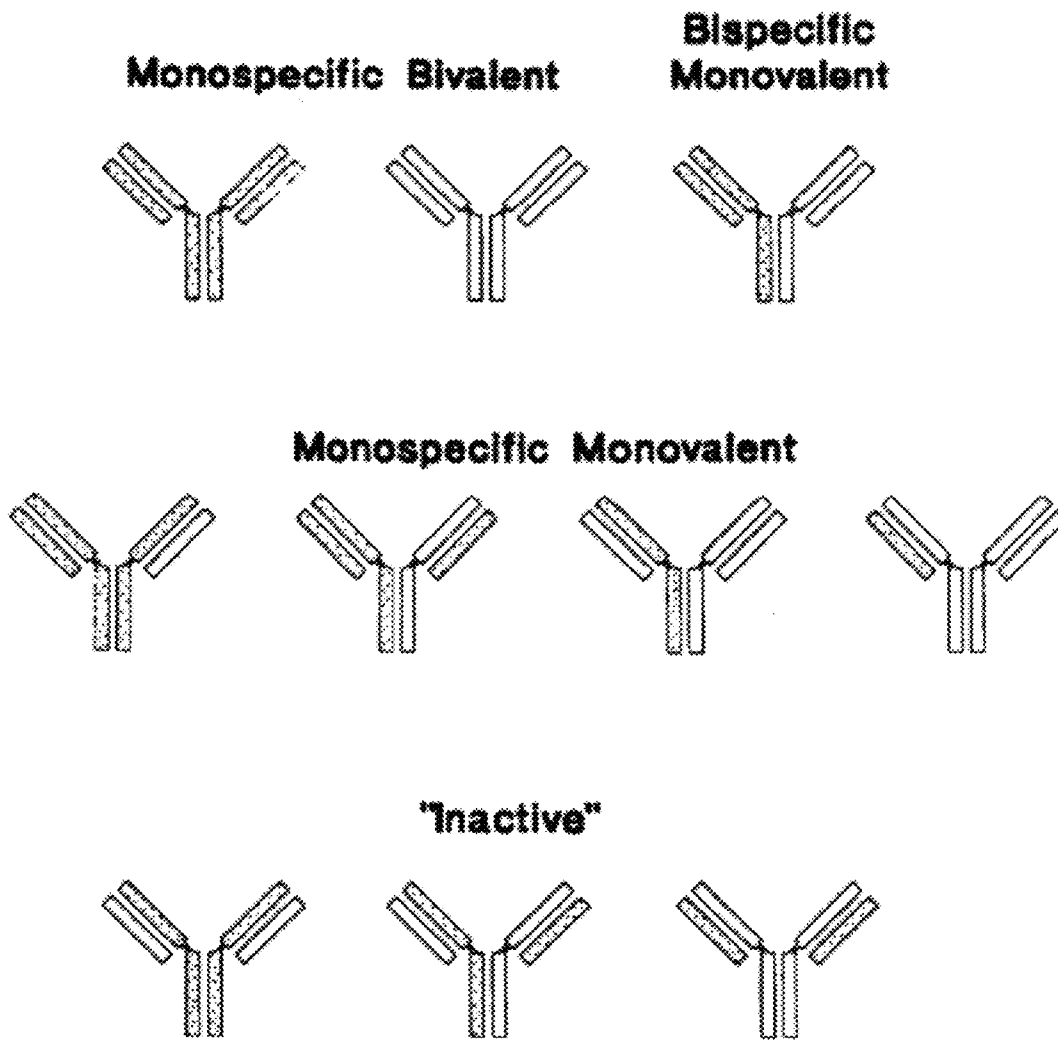


FIG. 1

BsF(ab')<sub>2</sub>  
CHEMICALLY COUPLED  
RODENT FRAGMENTS

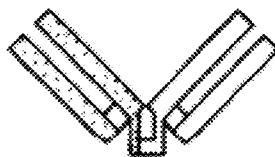


FIG. 2A

BsF(ab')<sub>2</sub>  
CHEMICALLY COUPLED  
E. coli DERIVED FRAGMENTS

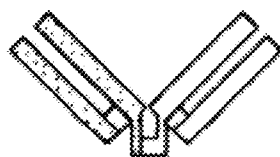


FIG. 2B

BsF(ab')<sub>2</sub>  
LEUCINE ZIPPER  
ASSEMBLED

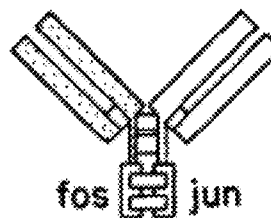
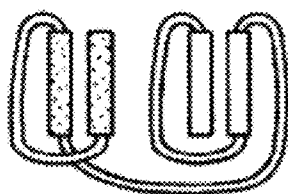


FIG. 2C



sFv DIMER

FIG. 2E



DIABODY

FIG. 2D

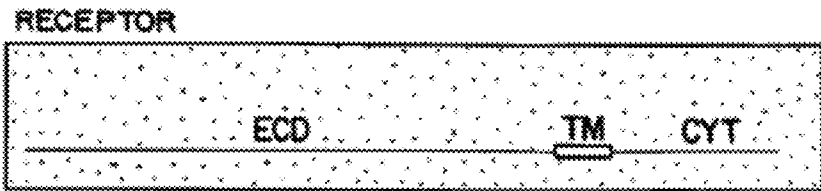


FIG. 3A

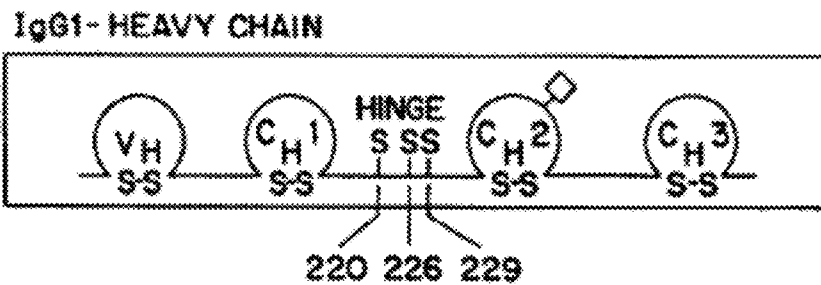


FIG. 3B

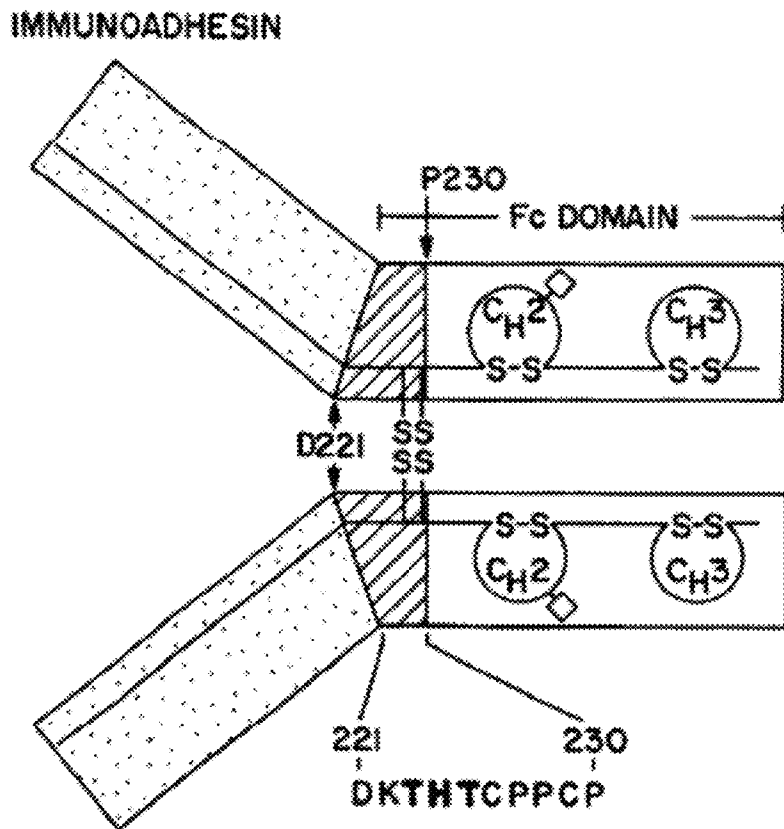


FIG. 3C

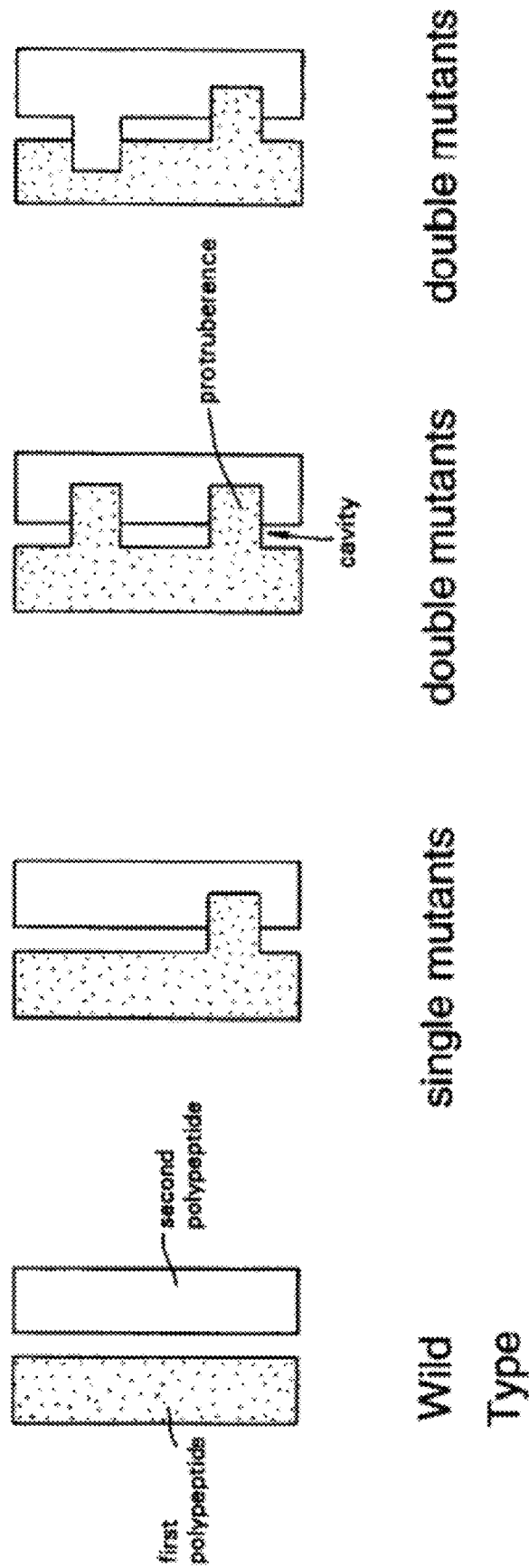


FIG. 4



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EDGE
361 hIgG1 G - Q P R E P Q V Y T L P P S R E E - - M T K N Q V S L T C
hIgG2 G - Q P R E P Q V Y T L P P S R E E - - M T K N Q V S L T C
hIgG3 G - Q P R Z P Q V Y T L P P S R E E - - M T K N Q V S L T C
hIgG4 G - Q P R E P Q V Y T L P P S R E E - - M T K N Q V S L T C

361 mIgG1 G - R P K A P Q V Y T I P P K E Q - - M A K D K V S L T C
mIgG2A G - P V R A P Q V Y V L P P P - A E E - - M T K K Z F S L T C
mIgG2B G - L V R A P Q V Y T L P P P - A E Q - - L S R K K D V S L T C
mIgG3 G - R A Q T P Q V Y T I P P P - R E Q - - M S K K K V S L T C
*****

400 hIgG1 L V K G F Y P S D - - I A V E W E S - B D - G Z P Z B N Y K
hIgG2 L V K G F Y P S D - - I A V E W E S - N G - - Q P E N N Y K
hIgG3 L V K G F Y P S D - - I A V E W E S - S G - - Q P E N N Y N
hIgG4 L V K G F Y P S D - - I A V E W Z S - N G - - Q P E N N Y K

mIgG1 M I T D F F P P E D I T V - - E W Q W - B G - - Q P A E N Y K
mIgG2A M I T G F F L P P A D I A V - - E W T S - N G - - R T E E N Y K
mIgG2B L V V G F F L P P A D I S V - - E W T S - N G - - H T E E N Y K
mIgG3 L V T N F F L P P S E A I S V - - E W T R - N G - - E L E Q D Y K
*****
MIDDLE
380
390
410

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FIG. 6A



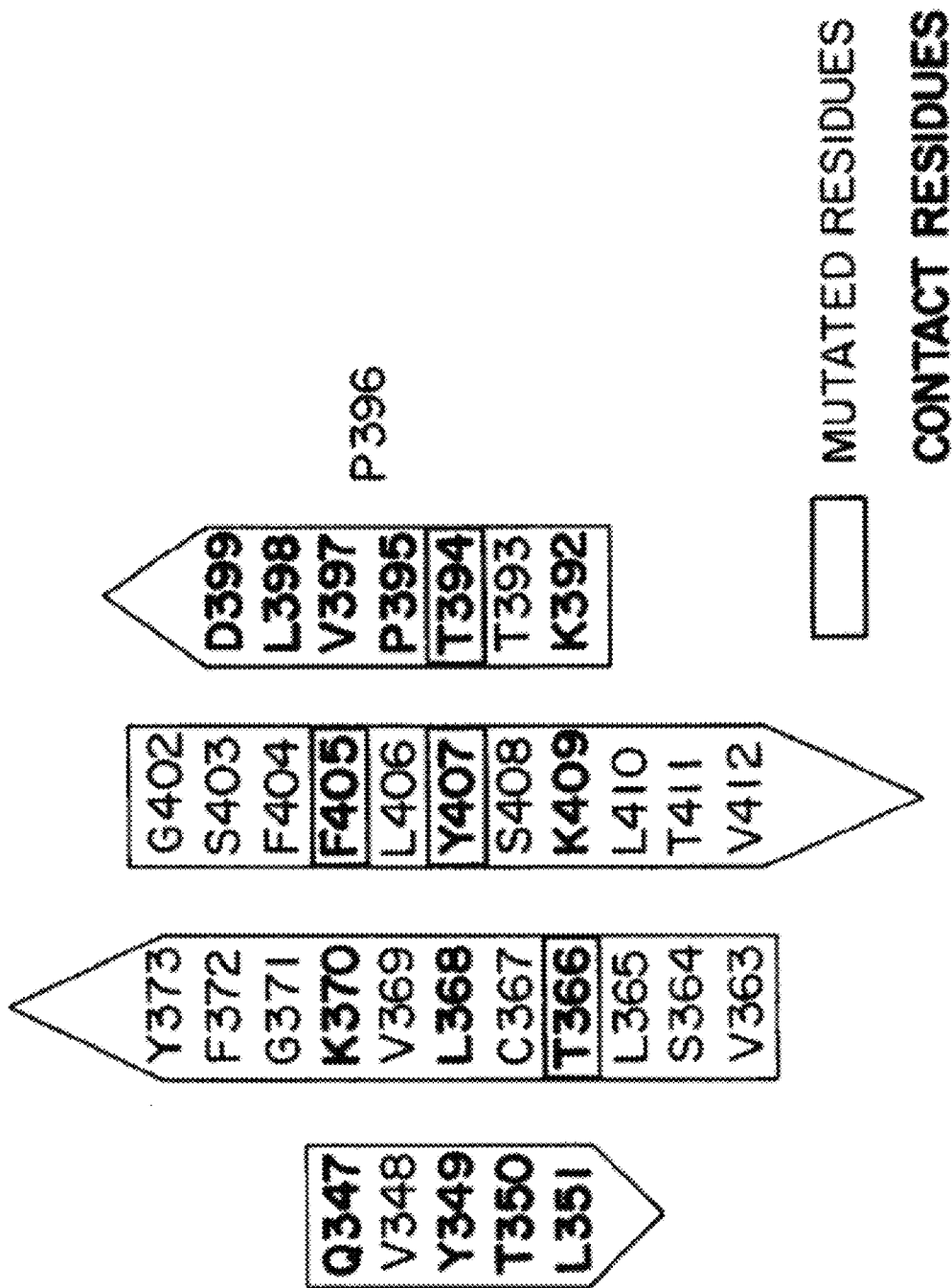


FIG. 7

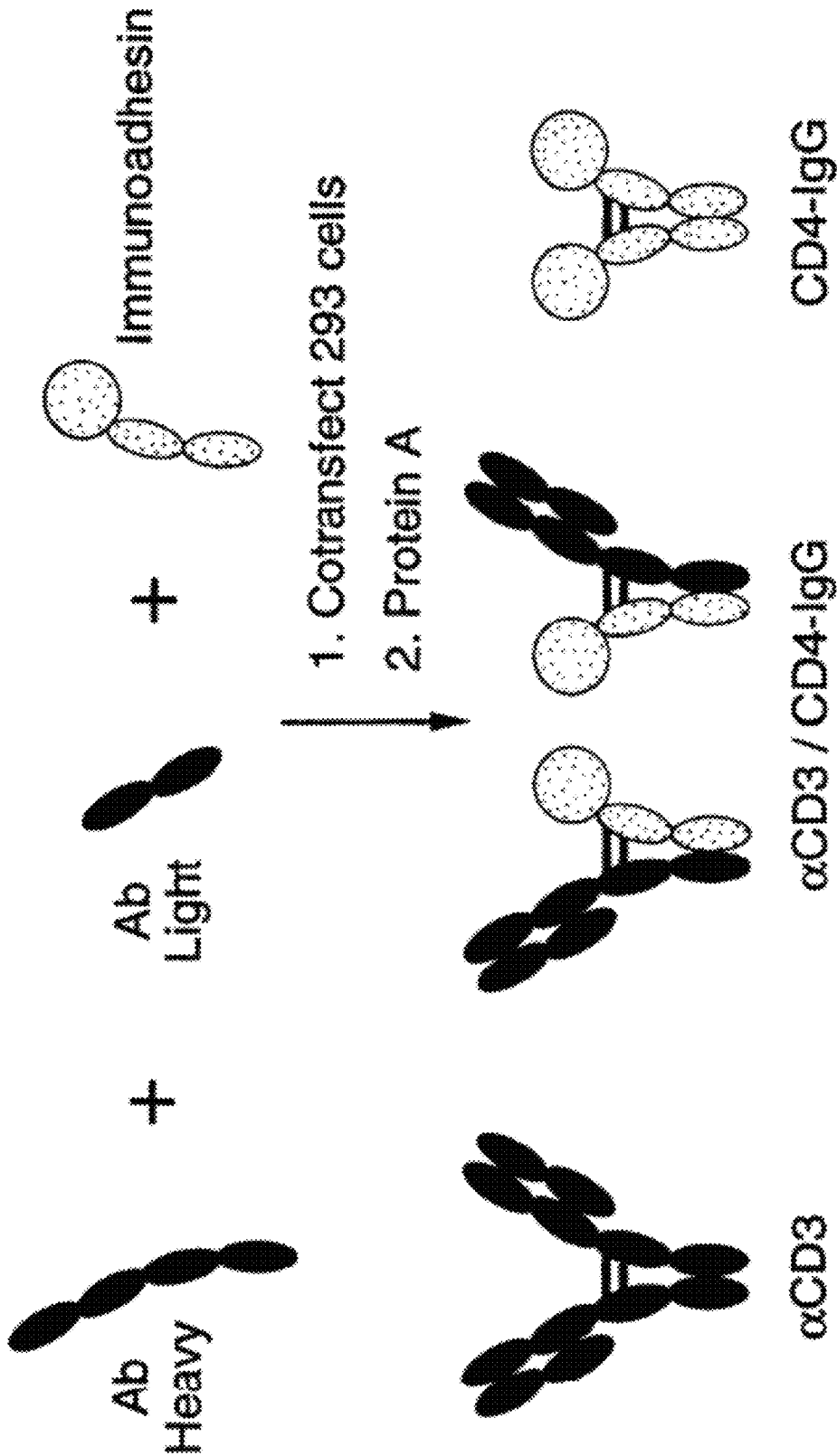


FIG.8

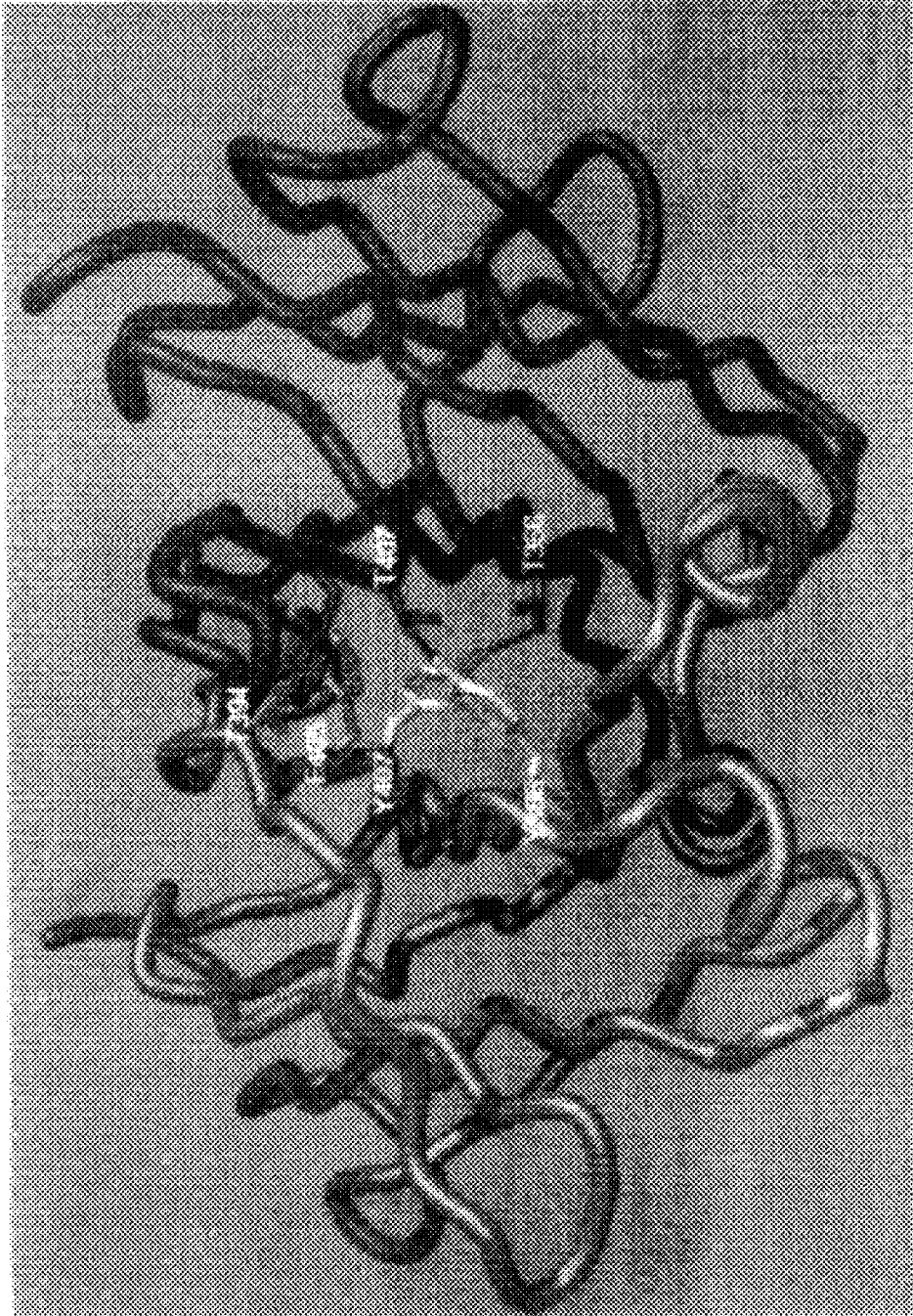
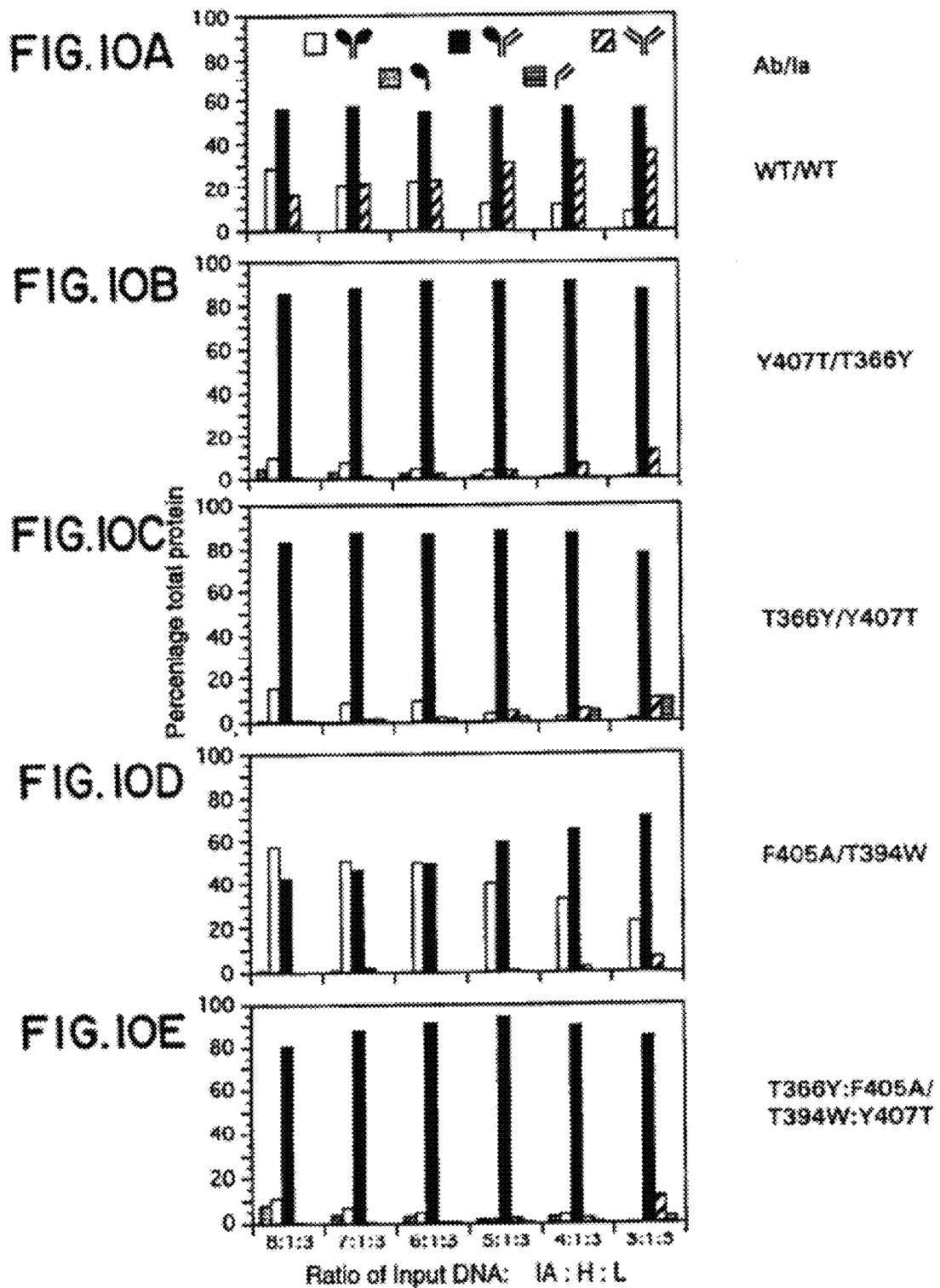


FIG. 9



## KNOB AND HOLES HETEROMERIC POLYPEPTIDES

### RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 11/533,709 filed Sep. 20, 2006 (now U.S. Pat. No. 7,695,936 issued Apr. 13, 2010), which is a continuation of U.S. application Ser. No. 10/010,245 filed Dec. 7, 2001 (now U.S. Pat. No. 7,642,228 issued Jan. 5, 2010), which is a continuation of application Ser. No. 08/974,183 filed Nov. 19, 1997, now abandoned, which is a continuation of U.S. application Ser. No. 08/399,106 filed Mar. 1, 1995 (now U.S. Pat. No. 5,731,168 issued Mar. 24, 1998), all of which are incorporated herein by reference and to which priority is claimed under 35 USC §120.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

This invention relates to a method for making heteromultimeric polypeptides such as multispecific antibodies (e.g. bispecific antibodies), multispecific immunoadhesins (e.g. bispecific immunoadhesins) as well as antibody-immunoadhesin chimeras and the heteromultimeric polypeptides made using the method.

#### 2. Description of Related Art Bispecific Antibodies

Bispecific antibodies (BsAbs) which have binding specificities for at least two different antigens have significant potential in a wide range of clinical applications as targeting agents for in vitro and in vivo immunodiagnosis and therapy, and for diagnostic immunoassays.

In the diagnostic areas, bispecific antibodies have been very useful in probing the functional properties of cell surface molecules and in defining the ability of the different Fc receptors to mediate cytotoxicity (Fanger et al., *Crit. Rev. Immunol.* 12:101-124 [1992]). Nolan et al., *Biochem. Biophys. Acta.* 1040:1-11 (1990) describe other diagnostic applications for BsAbs. In particular, BsAbs can be constructed to immobilize enzymes for use in enzyme immunoassays. To achieve this, one arm of the BsAb can be designed to bind to a specific epitope on the enzyme so that binding does not cause enzyme inhibition, the other arm of the BsAb binds to the immobilizing matrix ensuring a high enzyme density at the desired site. Examples of such diagnostic BsAbs include the rabbit anti-IgG/anti-ferritin BsAb described by Hammerling et al., *J. Exp. Med.* 128:1461-1473 (1968) which was used to locate surface antigens. BsAbs having binding specificities for horse radish peroxidase (HRP) as well as a hormone have also been developed. Another potential immunochemical application for BsAbs involves their use in two-site immunoassays. For example, two BsAbs are produced binding to two separate epitopes on the analyte protein—one BsAb binds the complex to an insoluble matrix, the other binds an indicator enzyme (see Nolan et al., supra).

Bispecific antibodies can also be used for in vitro or in vivo immunodiagnosis of various diseases such as cancer (Song-sivilai et al., *Clin. Exp. Immunol.* 79:315 [1990]). To facilitate this diagnostic use of the BsAb, one arm of the BsAb can bind a tumor associated antigen and the other arm can bind a detectable marker such as a chelator which tightly binds a radionuclide. Using this approach, Le Doussal et al. made a BsAb useful for radioimmunodetection of colorectal and thyroid carcinomas which had one arm which bound a carcinoembryonic antigen (CEA) and another arm which bound diethylenetriaminepentaacetic acid (DTPA). See Le Doussal et

al., *Int. J. Cancer Suppl.* 7:58-62 (1992) and Le Doussal et al., *J. Nucl. Med.* 34:1662-1671 (1993). Stickney et al. similarly describe a strategy for detecting colorectal cancers expressing CEA using radioimmunodetection. These investigators describe a BsAb which binds CEA as well as hydroxyethylthiourea-benzyl-EDTA (EOTUBE). See Stickney et al., *Cancer Res.* 51:6650-6655 (1991).

Bispecific antibodies can also be used for human therapy in redirected cytotoxicity by providing one arm which binds a target (e.g. pathogen or tumor cell) and another arm which binds a cytotoxic trigger molecule, such as the T-cell receptor or the Fcγ receptor. Accordingly, bispecific antibodies can be used to direct a patient's cellular immune defense mechanisms specifically to the tumor cell or infectious agent. Using this strategy, it has been demonstrated that bispecific antibodies which bind to the Fcγ RIII (i.e. CD16) can mediate tumor cell killing by natural killer (NK) cell/large granular lymphocyte (LGL) cells in vitro and are effective in preventing tumor growth in vivo. Segal et al., *Chem. Immunol.* 47:179 (1989) and Segal et al., *Biologic Therapy of Cancer* 2(4) DeVita et al. eds. J. B. Lippincott, Philadelphia (1992) p. 1. Similarly, a bispecific antibody having one arm which binds FcγRIII and another which binds to the HER2 receptor has been developed for therapy of ovarian and breast tumors that overexpress the HER2 antigen. (Hseih-Ma et al. *Cancer Research* 52:6832-6839 [1992] and Weiner et al. *Cancer Research* 53:94-100 [1993]). Bispecific antibodies can also mediate killing by T cells. Normally, the bispecific antibodies link the CD3 complex on T cells to a tumor-associated antigen. A fully humanized F(ab')<sub>2</sub> BsAb consisting of anti-CD3 linked to anti-p185<sup>HER2</sup> has been used to target T cells to kill tumor cells overexpressing the HER2 receptor. Shalaby et al., *J. Exp. Med.* 175(1):217 (1992). Bispecific antibodies have been tested in several early phase clinical trials with encouraging results. In one trial, 12 patients with lung, ovarian or breast cancer were treated with infusions of activated T-lymphocytes targeted with an anti-CD3/anti-tumor (MOC31) bispecific antibody. deLeij et al. *Bispecific Antibodies and Targeted Cellular Cytotoxicity*, Romet-Lemonne, Fanger and Segal Eds., Lienhart (1991) p. 249. The targeted cells induced considerable local lysis of tumor cells, a mild inflammatory reaction, but no toxic side effects or anti-mouse antibody responses. In a very preliminary trial of an anti-CD3/anti-CD19 bispecific antibody in a patient with B-cell malignancy, significant reduction in peripheral tumor cell counts was also achieved. Clark et al. *Bispecific Antibodies and Targeted Cellular Cytotoxicity*, Romet-Lemonne, Fanger and Segal Eds., Lienhart (1991) p. 243. See also Kroesen et al., *Cancer Immunol. Immunother.* 37:400-407 (1993), Kroesen et al., *Br. J. Cancer* 70:652-661 (1994) and Weiner et al., *J. Immunol.* 152:2385 (1994) concerning therapeutic applications for BsAbs.

Bispecific antibodies may also be used as fibrinolytic agents or vaccine adjuvants. Furthermore, these antibodies may be used in the treatment of infectious diseases (e.g. for targeting of effector cells to virally infected cells such as HIV or influenza virus or protozoa such as *Toxoplasma gondii*), used to deliver immunotoxins to tumor cells, or target immune complexes to cell surface receptors (see Fanger et al., supra).

Use of BsAbs has been effectively stymied by the difficulty of obtaining BsAbs in sufficient quantity and purity. Traditionally, bispecific antibodies were made using hybrid-hybridoma technology (Millstein and Cuello, *Nature* 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody mol-

ecules, of which only one has the correct bispecific structure (see FIG. 1 herein). The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Accordingly, techniques for the production of greater yields of BsAb have been developed. These are depicted in FIGS. 2A-2E herein. As shown in FIG. 2A, bispecific antibodies can be prepared using chemical linkage. To achieve chemical coupling of antibody fragments, Brennan et al., *Science* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')<sub>2</sub> fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the BsAb. The BsAbs produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies (see FIG. 2B). Shalaby et al., *J. Exp. Med.* 175:217-225 (1992) describe the production of a fully humanized BsAb F(ab')<sub>2</sub> molecule having one arm which binds p185<sup>HER2</sup> and another arm which binds CD3. Each Fab' fragment was separately secreted from *E. coli*, and subjected to directed chemical coupling in vitro to form the BsAb. The BsAb thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets. See also Rodrigues et al., *Int. J. Cancers* (Suppl.) 7:45-50 (1992).

Various techniques for making and isolating BsAb fragments directly from recombinant cell cultures have also been described. For example, bispecific F(ab')<sub>2</sub> heterodimers have been produced using leucine zippers (see FIG. 2C). Kostelny et al., *J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of anti-CD3 and anti-interleukin-2 receptor (IL-2R) antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then reoxidized to form the antibody heterodimers. The BsAbs were found to be highly effective in recruiting cytotoxic T cells to lyse HuT-102 cells in vitro. The advent of the "diabody" technology described by Hollinger et al., *PNAS (USA)* 90:6444-6448 (1993) has provided an alternative mechanism for making BsAb fragments. The fragments comprise a heavy chain variable domain (V<sub>H</sub>) connected to a light chain variable domain (V<sub>L</sub>) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V<sub>H</sub> and V<sub>L</sub> domains of one fragment are forced to pair with the complementary V<sub>L</sub> and V<sub>H</sub> domains of another fragment, thereby forming two antigen-binding sites (see FIG. 2D herein). Another strategy for making BsAb fragments by the use of single chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.* 152: 5368 (1994). These researchers designed an antibody which comprised the V<sub>H</sub> and V<sub>L</sub> domains of an antibody directed against the T cell receptor joined by a 25 amino acid residue linker to the V<sub>H</sub> and V<sub>L</sub> domains of an anti-fluorescein antibody. The refolded molecule (see FIG. 2E herein) bound to fluorescein and the T cell receptor and redirected the lysis of human tumor cells that had fluorescein covalently linked to their surface.

It is apparent that several techniques for making bispecific antibody fragments which can be recovered directly from

recombinant cell culture have been reported. However, full length BsAbs may be preferable to BsAb fragments for many clinical applications because of their likely longer serum half-life and possible effector functions.

#### 5 Immunoadhesins

Immunoadhesins (Ia's) are antibody-like molecules which combine the binding domain of a protein such as a cell-surface receptor or a ligand (an "adhesin") with the effector functions of an immunoglobulin constant domain. Immunoadhesins can possess many of the valuable chemical and biological properties of human antibodies. Since immunoadhesins can be constructed from a human protein sequence with a desired specificity linked to an appropriate human immunoglobulin hinge and constant domain (Fc) sequence, the binding specificity of interest can be achieved using entirely human components. Such immunoadhesins are minimally immunogenic to the patient, and are safe for chronic or repeated use.

Immunoadhesins reported in the literature include fusions of the T cell receptor (Gascoigne et al., *Proc. Natl. Acad. Sci. USA* 84:2936-2940 [1987]); CD4 (Capon et al., *Nature* 337: 525-531 [1989]; Trauneker et al., *Nature* 339:68-70 [1989]; Zettmeissl et al., *DNA Cell Biol. USA* 9:347-353 [1990]; and Bym et al., *Nature* 344:667-670 [1990]); L-selectin or homing receptor (Watson et al., *J. Cell. Biol.* 110:2221-2229 [1990]; and Watson et al., *Nature* 349:164-167 [1991]); CD44 (Aruffo et al., *Cell* 61:1303-1313 [1990]); CD28 and B7 (Linsley et al., *J. Exp. Med.* 173:721-730 [1991]); CTLA-4 (Lisley et al., *J. Exp. Med.* 174:561-569 [1991]); CD22 (Stamenkovic et al., *Cell* 66:1133-1144 [1991]); TNF receptor (Ashkenazi et al., *Proc. Natl. Acad. Sci. USA* 88:10535-10539 [1991]; Lesslauer et al., *Eur. J. Immunol.* 27:2883-2886 [1991]; and Peppel et al., *J. Exp. Med.* 174:1483-1489 [1991]); NP receptors (Bennett et al., *J. Biol. Chem.* 266: 23060-23067 [1991]); interferon  $\gamma$  receptor (Kurschner et al., *J. Biol. Chem.* 267:9354-9360 [1992]); 4-1BB (Chalupny et al., *PNAS [USA]* 89:10360-10364 [1992]) and IgE receptor  $\gamma$  (Ridgway and Gorman, *J. Cell. Biol.* Vol. 115, Abstract No. 1448 [1991]).

Examples of immunoadhesins which have been described for therapeutic use include the CD4-IgG immunoadhesin for blocking the binding of HIV to cell-surface CD4. Data obtained from Phase I clinical trials in which CD4-IgG was administered to pregnant women just before delivery suggests that this immunoadhesin may be useful in the prevention of maternal-fetal transfer of HIV. Ashkenazi et al., *Intern. Rev. Immunol.* 10:219-227 (1993). An immunoadhesin which binds tumor necrosis factor (TNF) has also been developed. TNF is a proinflammatory cytokine which has been shown to be a major mediator of septic shock. Based on a mouse model of septic shock, a TNF receptor immunoadhesin has shown promise as a candidate for clinical use in treating septic shock (Ashkenazi et al., supra). Immunoadhesins also have non-therapeutic uses. For example, the L-selectin receptor immunoadhesin was used as a reagent for histochemical staining of peripheral lymph node high endothelial venules (HEV). This reagent was also used to isolate and characterize the L-selectin ligand (Ashkenazi et al., supra).

If the two arms of the immunoadhesin structure have different specificities, the immunoadhesin is called a "bispecific immunoadhesin" by analogy to bispecific antibodies. Dietsch et al., *J. Immunol. Methods* 162:123 (1993) describe such a bispecific immunoadhesin combining the extracellular domains of the adhesion molecules, E-selectin and P-selectin. Binding studies indicated that the bispecific immunoglobulin fusion protein so formed had an enhanced ability to bind to a

myeloid cell line compared to the monospecific immunoadhesins from which it was derived.

#### Antibody-Immunoadhesin Chimeras

Antibody-immunoadhesin (Ab/Ia) chimeras have also been described in the literature. These molecules combine the binding region of an immunoadhesin with the binding domain of an antibody.

Berg et al., *PNAS (USA)* 88:4723-4727 (1991) made a bispecific antibody-immunoadhesin chimera which was derived from murine CD4-IgG. These workers constructed a tetrameric molecule having two arms. One arm was composed of CD4 fused with an antibody heavy-chain constant domain along with a CD4 fusion with an antibody light-chain constant domain. The other arm was composed of a complete heavy-chain of an anti-CD3 antibody along with a complete light-chain of the same antibody. By virtue of the CD4-IgG arm, this bispecific molecule binds to CD3 on the surface of cytotoxic T cells. The juxtaposition of the cytotoxic cells and HIV-infected cells results in specific killing of the latter cells.

While Berg et al. describe a bispecific molecule that was tetrameric in structure, it is possible to produce a trimeric hybrid molecule that contains only one CD4-IgG fusion. See Chamow et al., *J. Immunol.* 153:4268 (1994). The first arm of this construct is formed by a humanized anti-CD3  $\gamma$  light chain and a humanized anti-CD3  $\gamma$  heavy chain. The second arm is a CD4-IgG immunoadhesin which combines part of the extracellular domain of CD4 responsible for gp120 binding with the Fc domain of IgG. The resultant Ab/Ia chimera mediated killing of HIV-infected cells using either pure cytotoxic T cell preparations or whole peripheral blood lymphocyte (PBL) fractions that additionally included Fc receptor-bearing large granular lymphocyte effector cells.

In the manufacture of the above-mentioned heteromultimers, it is desirable to increase the yields of the desired heteromultimer over the homomultimer(s). The invention described herein provides a means for achieving this.

#### SUMMARY OF THE INVENTION

This application describes a "protuberance-into-cavity" strategy which serves to engineer an interface between a first and second polypeptide for hetero-oligomerization. See FIG. 4 for a schematic illustration of the strategy employed. The preferred interface comprises at least a part of the  $C_{H3}$  domain of an antibody constant domain. "Protuberances" are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the protuberances are optionally created on the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). Where a suitably positioned and dimensioned protuberance or cavity exists at the interface of either the first or second polypeptide, it is only necessary to engineer a corresponding cavity or protuberance, respectively, at the adjacent interface.

Accordingly, the invention can be said to relate to a method of preparing a heteromultimer comprising a first polypeptide and a second polypeptide which meet at an interface, wherein the first polypeptide has a protuberance at the interface thereof which is positionable in a cavity at the interface of the second polypeptide. In one aspect, the method involves: (a) culturing a host cell comprising nucleic acid encoding the first polypeptide and second polypeptide, wherein the nucleic acid encoding the first polypeptide has been altered from the original nucleic acid to encode the protuberance or the nucleic acid encoding the second polypeptide has been

altered from the original nucleic acid to encode the cavity, or both, such that the nucleic acid is expressed; and (b) recovering the heteromultimer from the host cell culture.

Normally, the nucleic acid encoding both the first polypeptide and the second polypeptide are altered to encode the protuberance and cavity, respectively. Preferably the first and second polypeptides each comprise an antibody constant domain such as the  $C_{H3}$  domain of a human IgG<sub>1</sub>.

The invention also provides a heteromultimer (such as a bispecific antibody, bispecific immunoadhesin or antibody/immunoadhesin chimera) comprising a first polypeptide and a second polypeptide which meet at an interface. The interface of the first polypeptide comprises a protuberance which is positionable in a cavity in the interface of the second polypeptide, and the protuberance or cavity, or both, have been introduced into the interface of the first and second polypeptides respectively. The heteromultimer may be provided in the form of a composition further comprising a pharmaceutically acceptable carrier.

The invention also relates to a host cell comprising nucleic acid encoding the heteromultimer of the preceding paragraph wherein the nucleic acid encoding the first polypeptide and second polypeptide is present in a single vector or in separate vectors. The host cell can be used in a method of making a heteromultimer which involves culturing the host cell so that the nucleic acid is expressed and recovering the heteromultimer from the cell culture.

In yet a further aspect, the invention provides a method of preparing a heteromultimer comprising:

(a) altering a first nucleic acid encoding a first polypeptide so that an amino acid residue in the interface of the first polypeptide is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance on the first polypeptide;

(b) altering a second nucleic acid encoding a second polypeptide so that an amino acid residue in the interface of the second polypeptide is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity in the second polypeptide, wherein the protuberance is positionable in the cavity;

(c) introducing into a host cell the first and second nucleic acids and culturing the host cell so that expression of the first and second nucleic acid occurs; and

(d) recovering the heteromultimer formed from the cell culture.

The invention provides a mechanism for increasing the yields of the heteromultimer over other unwanted end-products such as homomultimers. Preferably, the yields of the heteromultimer recovered from recombinant cell culture are at least greater than 80% and preferably greater than 90% compared to the by-product homomultimer(s).

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the various antibody molecules which may be generated when the traditional hybrid-hybridoma technique of Millstein and Cuello, supra, is used for making full length BsAbs.

FIGS. 2A-2E illustrate the various techniques of the background art for manufacturing BsAb fragments, reviewed in the background section above.

FIGS. 3A-3C depict an exemplary strategy for making an immunoadhesin dimer (SEQ ID NO.:1) (FIG. 3C) comprising the binding domain of a receptor (FIG. 3A) and the constant domain of an IgG<sub>1</sub> immunoglobulin (FIG. 3B).

FIG. 4 illustrates schematically the protuberance-into-cavity strategy of the instant application for generating heteromultimers.

FIG. 5 shows the interface residues of the  $C_H3$  domain of the immunoglobulins IgG (SEQ ID NO.:2), IgA (SEQ ID NO.:3), IgD (SEQ ID NO.:4), IgE (SEQ ID NO.:5) and IgM (SEQ ID NO.:6). The  $C_H3$  domain of each of these immunoglobulins is made up of a “ $\bar{y}$ -sandwich”, which is comprised of two separate and parallel “ $\bar{y}$ -sheets”. One of the  $\bar{y}$ -sheets provides the interface residues, the other is the “exterior  $\bar{y}$ -sheet”. The  $\bar{y}$ -sheet forming the interface is formed from four “ $\bar{y}$ -strands”. The residues of each of the seven  $\bar{y}$ -strands of the  $C_H3$  domain of the various immunoglobulins are identified by dashed overlining. The residues in the middle and edge  $\bar{y}$ -strands of the interface are identified, as are those of the exterior  $\bar{y}$ -sheet. Residue numbering is according to Fc crystal structure (Deisenhofer, *Biochem.* 20:2361 [1981]). The residues buried in the interior of the  $C_H3$  domain are identified with a “B”, those which are partially buried in the interior of the  $C_H3$  domain are identified with a “b”, those “contact” residues which are partially buried at the interface (i.e. 26%-10% exposed) are identified with an “i” and those which are buried at the interface (i.e. <6% exposed) are identified with an “l”. The bold residues are optimal candidate original residues for replacement with import residues.

FIG. 6 identifies the interface residues of human (h) ‘hIgG1’, ‘hIgG2’, ‘hIgG3’, ‘hIgG4’, and ‘hIgE’ disclosed as SEQ ID NOs.:7-10 and 15, respectively, in order of appearance) or murine (m) (‘mIgG1’, ‘mIgG2A’, ‘mIgG2B’ and ‘mIgG3’, disclosed as SEQ ID NOs.:11-14, respectively, in order of appearance) IgG subtypes (B=ASX and Z=GLX). The residues in  $\bar{y}$ -strands at the edge and middle of the interface are bracketed and “contact” residues are indicated with arrows. Sequences obtained from Miller et al., *J. Mol. Biol.* 216:965 (1990) and Kabat et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md., ed. 5, (1991). It is apparent that the contact residues are highly conserved.

FIG. 7 shows the interface residues of the  $C_H3$  domain of human IgG<sub>1</sub> SEQ ID NOs.:16-19, respectively, in order of appearance). Data derived from Miller et al., *J. Mol. Biol.* 216:965 (1990). “Contact” residues are shown and those residues mutated in the examples described herein are boxed.

FIG. 8 shows schematically the co-transfection assay for examining Fc heterodimerization described in the examples.

FIG. 9 depicts a  $C_H3$  dimer based upon a 2.9 Å structure of human IgG<sub>1</sub> Fc (Deisenhofer, *Biochem.* 20:2361 [1981]) highlighting T366Y and Y407T mutations on opposite sides of the interface together with residues Phe<sup>405</sup> and Thr<sup>394</sup> (“Kabat numbering”—Kabat et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md., ed. 5, [1991]).

FIGS. 10A-10E depict a scanning densitometric analysis of SDS-PAGE of products from co-transfection of antibody (Ab) heavy (H) and light (L) chains with immunoadhesin (Ia). FIG. 10A shows wild-type; FIG. 10B shows mutant Ab Y407T, Ia T366Y; FIG. 10C shows mutant Ab T366Y, Ia Y407T; FIG. 10D shows mutant Ab F405A, Ia T394W; and FIG. 10E shows mutant Ab T366Y:F405A, Ia T394W:Y407T. Data presented are the mean from at least 2 independent experiments. The densitometric signal response was found to be linear (R=0.9993) over the experimental range used (0.02-10 $\bar{y}$ g) as judged by control experiment using a closely related humanized antibody, huMAb4D5-8 (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285 [1992]).

## I. Definitions

In general, the following words or phrases have the indicated definitions when used in the description, examples, and claims:

A “heteromultimer” or “heteromultimeric polypeptide” is a molecule comprising at least a first polypeptide and a second polypeptide, wherein the second polypeptide differs in amino acid sequence from the first polypeptide by at least one amino acid residue. Preferably, the heteromultimer has binding specificity for at least two different ligands or binding sites. The heteromultimer can comprise a “heterodimer” formed by the first and second polypeptide or can form higher order tertiary structures where polypeptides in addition to the first and second polypeptide are present. Exemplary structures for the heteromultimer include heterodimers (e.g. the bispecific immunoadhesin described by Dietsch et al., supra), heterotrimers (e.g. the Ab/Ia chimera described by Chamow et al., supra), heterotetramers (e.g. a bispecific antibody) and further oligomeric structures.

As used herein, “polypeptide” refers generally to peptides and proteins having more than about ten amino acids. Preferably, mammalian polypeptides (polypeptides that were originally derived from a mammalian organism) are used, more preferably those which are directly secreted into the medium. Examples of bacterial polypeptides include, e.g., alkaline phosphatase and  $\bar{y}$ -lactamase. Examples of mammalian polypeptides include molecules such as renin, a growth hormone, including human growth hormone; bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIc, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellierian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3,-4,-5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- $\bar{y}$ ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF- $\bar{y}$ 1, TGF- $\bar{y}$ 2, TGF- $\bar{y}$ 3, TGF- $\bar{y}$ 4, or TGF- $\bar{y}$ 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD-3, CD-4, CD-8, and CD-19; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors;













































