

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Application of:	Carter <i>et al.</i>	Confirmation No.:	8234
Application No.:	12/700,618	Art Unit:	1649
Filed:	February 4, 2010	Examiner:	Gucker, Stephen
For:	A METHOD FOR MAKING HETEROMULTIMERIC POLYPEPTIDES	Attorney Docket No:	12279-514-999

**AMENDMENT AND RESPONSE UNDER 37 C.F.R. § 1.116**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

In response to the final Office Action mailed May 26, 2011 (hereinafter “the Office Action”), and in accordance with the Rules of Practice, please consider and enter the following remarks.

**Amendments to the Claims** are reflected in the listing of claims which begins on page 2.

**Remarks** begin on page 7 of this paper.

Also submitted herewith is a Notice of Appeal and a copy of Merchant *et al.*, *Nature Biotechnology* 16: 677-681 (1998), in Appendix A.

**AMENDMENT TO THE CLAIMS:**

This Listing of Claims will replace all prior versions, and listings, of claims in the application.

**Listing of Claims:**

1-38. (Canceled)

39. **(Currently amended)** A method of preparing a heteromultimer comprising a first polypeptide and a second polypeptide each comprising a C<sub>H</sub>3 antibody constant domain, wherein said polypeptides meet at an engineered interface within the C<sub>H</sub>3 domain, the method comprising the steps of:

(a) creating an engineered protuberance in the interface of ~~one of the two polypeptides~~ the first polypeptide by replacing at least one contact residue of said polypeptide within its C<sub>H</sub>3 domain;

(b) creating an engineered cavity in the interface of the ~~other second~~ second polypeptide by replacing at least one contact residue of said polypeptide within its C<sub>H</sub>3 domain, wherein said protuberance in the first polypeptide is positional in said cavity of the second polypeptide so as to form a protuberance-into-cavity mutant pair, and wherein the contact residue to be replaced on ~~each polypeptide~~ the first polypeptide corresponds to an IgG residue selected from the group consisting of amino acid residues 347, 349, 350, 351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5;

(c) expressing said first and second polypeptides in a host cell to obtain the heteromultimer;

(d) recovering the heteromultimer from the host cell culture;

wherein said creating an engineered protuberance comprises replacing the nucleic acid encoding the original residue with nucleic acid encoding an import residue having a larger side chain volume than the original residue; and

wherein said creating an engineered cavity comprises replacing the nucleic acid encoding an original residue with nucleic acid encoding an import residue having a smaller side chain volume than the original residue.

40. – 41. (Canceled)

42. **(Currently amended)** The method of claim 39, wherein the contact residue to be replaced on ~~each polypeptide~~ the first polypeptide is selected from the group consisting of amino acid residues 366, 368, 370, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5.
43. **(Previously presented)** The method of claim 39, wherein said engineered interface within the C<sub>H</sub>3 domain comprises at least two protuberance-into-cavity mutant pairs created by repeating steps (a) and (b).
44. **(Previously presented)** The method of claim 43, wherein said at least two protuberance-into-cavity mutant pairs are created by creating at least one protuberance and at least one cavity on the first polypeptide and creating at least one cavity and at least one protuberance on the second polypeptide.
45. **(Previously presented)** The method of claim 43, wherein said at least two protuberance-into-cavity mutant pairs are created by creating more than one protuberance on the first polypeptide and creating more than one cavity on the second polypeptide.
46. **(Previously presented)** The method of claim 39, wherein the C<sub>H</sub>3 antibody constant domain is from an immunoglobulin selected from the group consisting of IgG, IgA, IgD, IgE and IgM.
47. **(Previously presented)** The method of claim 46, wherein the immunoglobulin is IgG.
48. **(Previously presented)** The method of claim 47, wherein the IgG is of a subtype selected from the group consisting of IgG1, IgG2a, IgG2b, IgG3 and IgG4.
49. **(Previously presented)** The method of claim 47, wherein the IgG is a human IgG.
50. **(Previously presented)** The method of claim 39, wherein the heteromultimer is a bispecific antibody.
51. **(Previously presented)** The method of claim 39, wherein the heteromultimer is a bispecific immunoadhesin.
52. **(Previously presented)** The method of claim 39, wherein the heteromultimer is an

antibody-immunoadhesin chimera (Ab/Ia).

53. **(Previously presented)** The method of claim 52, wherein the Ab/Ia is anti-CD3/CD4-IgG.
54. **(Previously presented)** An isolated heteromultimer prepared by the method of claim 39.
55. **(Currently amended)** An isolated heteromultimer comprising a first polypeptide and a second polypeptide each comprising a C<sub>H</sub>3 antibody constant domain, said heteromultimer having the following characteristics:
- (a) said first and second polypeptides meet at an engineered interface within the C<sub>H</sub>3 domain;
  - (b) ~~one of the two polypeptides~~ the first polypeptide comprises at least one engineered protuberance in said interface, said protuberance comprising at least one altered contact residue;
  - (c) ~~the other~~ second polypeptide comprises at least one engineered cavity in ~~its~~ said interface, said cavity comprising at least one altered contact residue;
  - (d) said protuberance is positional in said cavity so as to form a protuberance-into-cavity mutant pair; and
  - (e) said at least one altered contact residue of ~~each of said protuberance and said cavity~~ the first polypeptide corresponds to an IgG residue selected from the group consisting of amino acid residues 347, 349, 350, 351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5.
56. **(Previously presented)** The heteromultimer of claim 55, wherein said protuberance comprises the replacement of the original contact residue with an import residue having a larger side chain volume.
57. **(Previously presented)** The heteromultimer of claim 55, wherein said cavity comprises the replacement of the original contact residue with an import residue having a smaller side chain volume.
58. **(Previously presented)** The heteromultimer of claim 55, wherein the altered contact residue is selected from the group consisting of amino acid residues 366, 368, 370, 405, 407

and 409, according to the amino acid numbering as shown in Figure 5.

59. **(Previously presented)** The heteromultimer of claim 55, wherein said engineered interface within the C<sub>H</sub>3 domain comprises at least two protuberance-into-cavity mutant pairs between the first and second polypeptides.

60. **(Previously presented)** The heteromultimer of claim 59, wherein said at least two protuberance-into-cavity mutant pairs comprise at least one protuberance and at least one cavity on the first polypeptide, and at least one cavity and at least one protuberance on the second polypeptide.

61. **(Previously presented)** The heteromultimer of claim 59, wherein said at least two protuberance-into-cavity mutant pairs comprise more than one protuberance on the first polypeptide and more than one cavity on the second polypeptide.

62. **(Previously presented)** The heteromultimer of claim 55, wherein the C<sub>H</sub>3 antibody constant domain is from an immunoglobulin selected from the group consisting of IgG, IgA, IgD, IgE and IgM.

63. **(Previously presented)** The heteromultimer of claim 62, wherein the immunoglobulin is IgG.

64. **(Previously presented)** The heteromultimer of claim 63, wherein the IgG is of a subtype selected from the group consisting of IgG1, IgG2a, IgG2b, IgG3 and IgG4.

65. **(Previously presented)** The heteromultimer of claim 63, wherein the IgG is a human IgG.

66. **(Previously presented)** The heteromultimer of claim 55, wherein the heteromultimer is a bispecific antibody.

67. **(Previously presented)** The heteromultimer of claim 55, wherein the heteromultimer is a bispecific immunoadhesin.

68. **(Previously presented)** The heteromultimer of claim 55, wherein the heteromultimer is an antibody-immunoadhesin chimera (Ab/Ia).

69. **(Previously presented)** The heteromultimer of claim 68, wherein the Ab/Ia is anti-

CD3/CD4-IgG.

70. **(Previously presented)** A composition comprising the heteromultimer of claim 55 and a pharmaceutically acceptable carrier.

71. **(Previously presented)** A host cell comprising nucleic acid encoding the heteromultimer of claim 55.

72. **(Previously presented)** The host cell of claim 71, wherein the nucleic acid encoding the first polypeptide and the nucleic acid encoding the second polypeptide are present in a single vector.

73. **(Previously presented)** The host cell of claim 71, wherein the nucleic acid encoding the first polypeptide and the nucleic acid encoding the second polypeptide are present in separate vectors.

74. **(Previously presented)** A method of making a heteromultimer comprising culturing the host cell of claim 71 so that the nucleic acid is expressed and recovering the heteromultimer from the cell culture.

75. **(Previously presented)** The method of claim 74, wherein the host cell is a mammalian cell.

76. **(Previously presented)** The method of claim 74, wherein the heteromultimer is recovered from the cell culture media.

### **REMARKS**

Previously, 39 and 42-76 were pending. In the present paper, claims 39, 42 and 55 are amended.

Upon entry of this Amendment. Claims 39 and 42-76 will be pending.

#### **I. AMENDMENTS TO THE CLAIMS**

Claim 39 has been amended to specify that the claimed method comprises the steps of: “creating an engineered protuberance in the interface of the first polypeptide by replacing at least one contact residue of said polypeptide within its C<sub>H3</sub> domain” and “creating an engineered cavity in the interface of the second polypeptide by replacing at least one contact residue of said polypeptide within its C<sub>H3</sub> domain.” Support for this amendment can be found, for example, at page 8, lines 12 to 21 of the specification.

Claim 39 has also been amended to recite, in relevant parts, “wherein the contact residue to be replaced on the first polypeptide corresponds to an IgG residue selected from the group consisting of amino acid residues 347, 349, 350, 351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5.” Support for this amendment can be found, for example, at page 8, lines 12 to 21, Figures 5-7 and the Example at page 57 to 63 of the specification.

Claim 42 has been amended to recite, in relevant parts, “[t]he method of claim 39, wherein the contact residue to be replaced on the first polypeptide is selected from the group consisting of amino acid residues 366, 368, 370, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5.” Support for this amendment can be found, for example, at page 8, lines 12 to 21, Figures 5-7 and the Example at page 57 to 63 of the specification.

Claim 55 has been amended to recite, in relevant parts, “the first polypeptide comprises at least one engineered protuberance in said interface, said protuberance comprising at least one altered contact residue” and “the second polypeptide comprises at least one engineered cavity in said interface, said cavity comprising at least one altered contact residue.” Support for this amendment can be found, for example, at page 8, line 26 to page 9, line 3 of the specification.

Claim 55 is further amended to specify that “at least one altered contact residue of the first polypeptide corresponds to an IgG residue selected from the group consisting of amino

acid residues 347, 349, 350, 351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5.” Support for this amendment can be found, for example, at page 8, lines 12 to 21, Figures 5-7 and the Example at page 57 to 63 of the specification.

Applicants submit that these amendments do not introduce any new matter and are fully supported by the application as filed. Applicants further note that the amendments to the claims narrow the number of issues remaining for appeal and therefore are suitable for entry under 37 C.F.R. § 1.116.

## **II. THE ENABLEMENT REJECTION SHOULD BE WITHDRAWN**

The Examiner maintains the rejection of claims 39 and 42-76 for lack of enablement under 35 U.S.C. § 112, first paragraph. Office Action at pages 2-5. Specifically, the Examiner alleges that the specification does not reasonably provide enablement for methods or products where only the first or second polypeptide is altered.

Without acquiescing to the propriety of the Examiner’s rejection and solely to expedite prosecution of the instant application, claim 39 has been amended to recite, in relevant parts, the steps of “(a) creating an engineered protuberance in the interface of *the first polypeptide* by replacing at least one contact residue of said polypeptide within its C<sub>H3</sub> domain” and “(b) creating an engineered cavity in the interface of the *second polypeptide* by replacing at least one contact residue of said polypeptide within its C<sub>H3</sub> domain, wherein said protuberance in the *first polypeptide* is positional in said cavity of the *second polypeptide*” Similarly, claim 55 has been amended to recite, in relevant parts, “(b) the *first polypeptide* comprises at least one engineered protuberance in said interface, said protuberance comprising at least one altered contact residue” and “(c) the *second polypeptide* comprises at least one engineered cavity in said interface, said cavity comprising at least one altered contact residue.” Thus, Applicants respectfully submit that the amended claims require that both the first and second polypeptides of the heterodimer be altered. Thus, the amendments render the rejection moot. Applicants therefore respectfully request that this aspect of the enablement rejection be withdrawn.

The Examiner also alleges that the specification does not provide adequate description or guidance for heteromultimers that do not involve specific pairings of specific amino acid residues by number. Office Action at page 4. The Examiner alleges that the claims recite specific amino acid residues that are not “contact” residues as taught by page 10 of the

specification and Figure 5, such as amino acid residues 350, 392, 395, 396, and 398, and, therefore, do not seem to be able to form protuberance and cavity pairing as disclosed by the specification. Office Action at page 5. The Examiner further alleges that no adequate disclosure is made as to what are the corresponding contact residues for IgG amino acid positions 347, 349-351, 368, 370, 392, 395-399 and 409 according to the amino acid numbering as shown in Figure 5, in order for one of skilled in the art to carry out and make the claimed subject matter. *Id.* Applicants respectfully traverse this aspect of the rejection.

Applicants have amended claim 39 to recite, in relevant parts, “wherein the contact residue to be replaced on the first polypeptide corresponds to an IgG residue selected from the group consisting of amino acid residues 347, 349, 350, 351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5.” Similarly, Applicants have amended claim 55 to recite, in relevant parts that, “at least one altered contact residue of the first polypeptide corresponds to an IgG residue selected from the group consisting of amino acid residues 347, 349, 350, 351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5.”

Applicants respectfully submit that the specification as filed provides adequate disclosure for one skilled in the art to determine the corresponding contact residues on the second polypeptide for IgG amino acid positions 347, 349, 350, 351, 368, 370, 392, 395-399 and 409, and to carry out and make the claimed subject matter as recited in the amended claims without undue experimentation.

The test for enablement is whether one reasonably skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *See, e.g., In re Wands*, 858 F.2d 731, 736-37 (Fed. Cir. 1988). “The specification need not explicitly teach those in the art to make and use the invention.” *Amgen Inc. v. Hoechst Marion Roussel*, 65 U.S.P.Q.2d 1385, 1400 (Fed. Cir. 2003). Further, the test for undue experimentation is not merely quantitative since a considerable amount of experimentation is permissible, so long as it is merely routine; the key word is “undue,” not “experimentation.” *See, e.g., Wands*, 858 F.2d at 736-37. Factors to be considered in determining if experimentation is undue include the so-called “Wands factors” that include: the quantity of experimentation necessary; the amount of direction or guidance presented; the presence or absence of working examples; the nature of the invention; the state of the prior art; the relative skill of those in the art; the predictability or

unpredictability of the art; and the breadth of the claims. *Wands*, 858 F.2d at 737.

Applicants respectfully submit that no undue experimentation is required to make and use the claimed subject matter, at the very least, based on the breadth of the claims, the guidance and working examples provided by the specification and the quantity of the experimentation required.

Applicants respectfully submit that the breadth of the claims do not support a finding of undue experimentation. The claims are directed to sixteen different contact residues. As noted by the Examiner, the specification discloses the specific corresponding contact residues for four of these residues (366, 394, 405, 407). Thus, there are only twelve contact amino acid residues (347, 349-351, 368, 370, 392, 395-399 and 409) for which the corresponding contact residues are not explicitly disclosed in the specification.

Applicants respectfully submit that the specification provides sufficient direction and guidance for one skilled in the art to determine the corresponding contact residues on the second polypeptide for the remaining twelve contact residues without undue experimentation.

The specification discloses that IgG amino acid positions 347, 349-351, 368, 370, 392, 395-399 and 409 are suitable for modification. In particular, Figure 7 of the specification depicts that IgG amino acid positions 347, 349-351, 368, 370, 392, 395-399 and 409 are contact residues within the C<sub>H</sub>3 region of IgG and shows the relative positions of these residues with respect to one another. Page 36, lines 10 to 17 and Figure 7. Thus, contrary to the Examiner's assertion, amino acid residues 350, 392, 395, 396 and 398 are contact residues within the C<sub>H</sub>3 region of IgG in light of Figure 7 and Figure 5 of the specification.

Applicants further submit that one skilled in the art could routinely determine potential corresponding contact residues for IgG amino acid positions 347, 349-351, 368, 370, 392, 395-399 and 409 based on the guidance provided by the specification. In particular, Applicants submit that one skilled in the art could predict potential corresponding contact residues for IgG amino acid positions 347, 349-351, 368, 370, 392, 395-399 and 409, based on the relative position of each contact residue with respect to one another according to Figure 7. For example, one skilled in the art would recognize that the contact residue at amino acid position 351 would most likely not pair with any of the contact residues at amino acid positions 392, 394-399, 405, 407 or 409 based on the position of these residues in Figure 7.

Further, the specification also discloses that molecular graphics modeling programs such as the Insight program, can be used to predict potential contact residue partners for amino acid positions 347, 349-351, 368, 370, 392, 395-399 and 409 and determine the effects of modification and substitution of the contact residue pairs. Specification at page 35, line 25 to page 36, line 9. As shown in Merchant *et al.*, *Nature Biotechnology* 16: 677-681 (1998), submitted herewith in Appendix A, computer molecular modeling programs have been successfully used to identify potentially suitable contact residue partners for modification within the C<sub>H3</sub> region of IgG and to predict the effects of such modifications. Merchant at page 677, second column and Figure 2.

Following identification of potential contact residue partners, the potential amino acids can then be routinely screened following the teaching of the specification to confirm the pairings. In particular, the specification discloses methods of creating variant polypeptides and exemplary amino acid substitutions as well as methods of expressing the variant polypeptides and heteromultimers products. Specification at page 36, line 22 to page 39, line 28; page 40, line 1 to page 51, line 19; and Table 3.

The specification further discloses methods for screening of the variant heteromultimers to select the optimal variant. Specification at page 39, lines 26 to 28. Indeed, a working example provides one such method for assaying the ability of variant polypeptides to form heteromultimers by co-transfection of the polypeptides into 293 cells and identification of resulting heteromultimers using electroblotting and amino terminal sequencing techniques. Specification at page 57 to 63. Thus, following the disclosure of the present application, the skilled artisan can routinely identify contact residue pairs and construct and test appropriate heteromultimers.

Finally, Applicants respectfully submit that the quantity of experimentation required to make and use the claimed subject matter does not support a finding of undue experimentation. As mentioned, the test for undue experimentation is not merely quantitative since a considerable amount of experimentation is permissible, so long as it is merely routine. *See, e.g., Wands*, 858 F.2d at 736-37. Undue experimentation is experimentation that would require a level of ingenuity beyond what is expected from one of ordinary skill in the field. *Fields v. Conover*, 443 F.2d 1386, 1390-91 (C.C.P.A. 1971).

Applicants respectfully submit that the techniques and methods used to make variant polypeptides and screen for their ability to form heteromultimers are routine. As disclosed in the specification, techniques used to make the variant polypeptides, such as oligonucleotide-

mediated mutagenesis are well known in the art. Specification at page 36, lines 22-24. The specification discloses that the nucleic acids encoding the variant polypeptides can be expressed using widely used cloning and expression vectors and prokaryotic and eukaryotic expression systems. Specification at pages 40-51. Applicants further submit that testing the potential corresponding contact residues to determine appropriate amino acid residue pairs requires only routine electroblotting and amino terminal sequencing techniques. Thus, Applicants respectfully submit that only routine techniques need to be used to confirm the corresponding contact residue pairs.

Given the breadth of the claims combined with the guidance provided by the specification and working examples, Applicants submit that the pending claims are enabled as only routine screening would be required to determine the corresponding contact residues on the second polypeptide for IgG amino acid positions 347, 349-351, 368, 370, 392, 395-399 and 409.

For at least the foregoing reasons, Applicants respectfully submit that the presently pending claims are fully enabled. Accordingly, Applicant respectfully requests that the rejection of the presently pending claims under 35 U.S.C. § 112, first paragraph be withdrawn.

### **III. THE REJECTION FOR INDEFINITENESS SHOULD BE WITHDRAWN**

Claims 39-76 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Office Action at page 7. In particular, the Examiner contends that the numbering system that the claims rely on to define the metes and bounds of the amino acid residues is not clear, and that Figure 5 does not show any numbering for IgG1, IgG2a, IgG2b, IgG3, IgG4 or Ab/la. Applicants respectfully traverse this rejection.

Applicants respectfully direct the Examiner to Figures 6A and 6B, which depict an alignment of the interface domain of the immunoglobulin constant domain of various IgG subtypes and designates contact residues using arrows. Applicants submit that one skilled in the art, in light of Figures 6A and 6B and Figure 5, would be able to deduce the positions for IgG1, IgG2a, IgG2b, IgG3, IgG4 or Ab/la that correspond to the IgG amino acid positions recited in the claims, and presented in Figure 5, based on the sequence homology of the constant domain across IgG subtypes and the highly conserved contact residues as depicted in Figures 6A and 6B. Based on sequence homology, one of ordinary skill in the art can readily determine the positions of IgG1, IgG2a, IgG2b, IgG3, IgG4 or Ab/la that correspond to

positions 347, 349-351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409 of IgG as shown in Figure 5.

The Examiner also contends that the Markush groups of claims 39 and 55 are vague and indefinite as the amino acid residues recited do not clearly form any “protuberance-into-cavity” mutant pair species. Office Action at page 6. Without acquiescing to the Examiner’s rejection and solely to expedite prosecution of the instant application, claim 39 has been amended to recite, in relevant parts, that “the contact residue to be replaced on *the first polypeptide* corresponds to an IgG residue selected from the group consisting of amino acid residues 347, 349-351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5” Similarly, Applicants have amended claim 55 to recite, in relevant parts that, “at least one altered contact residue of *the first polypeptide* corresponds to an IgG residue selected from the group consisting of amino acid residues 347, 349-351, 366, 368, 370, 392, 394, 395, 397, 398, 399, 405, 407 and 409, according to the amino acid numbering as shown in Figure 5.” Applicants respectfully submit that the amended claims recite only one residue of a “protuberance-into-cavity” mutant pair species. Further, Applicants respectfully submit that one of ordinary skill in the art need not know the precise protuberance-into-cavity pairings to understand the metes and bounds of the claims, since the claims recite a finite number of contact residues which can be replaced to create a protuberance or cavity, and, as disclosed above, the specification discloses how to arrive at a compatible residue pairing once an interface residue is identified. *See*, Section II, *supra*.

In view of the foregoing, Applicants respectfully submit that the claims are clear and definite under 35 U.S.C. § 112, second paragraph. Accordingly, withdrawal of the rejection is respectfully requested.

### CONCLUSION


Applicants believe that the claims of the instant amendment meet all of the conditions for patentability and are in condition for allowance. Accordingly, an indication of the same is respectfully requested. If any issues remain in connection with this application, the Examiner is respectfully invited to telephone the undersigned to discuss the same.

AMENDMENT UNDER 37 C.F.R. § 1.116  
U.S. Application No. 12/700,618

No fees are believed to be due in connection herewith. However, should the Commissioner determine otherwise, please charge the required fee to Jones Day Deposit Account No. 50-3013 (referencing 403545-999514).

Respectfully submitted,

Date: August 25, 2011

  
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For: Laura A. Coruzzi (Reg. No. 30,742)

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## APPENDIX A

**Merchant *et al.*, *Nature Biotechnology* 16: 677-681 (1998)**

# An efficient route to human bispecific IgG

A. Margaret Merchant<sup>1</sup>, Zhenping Zhu<sup>1,2</sup>, Jean Q. Yuan<sup>3</sup>, Audrey Goddard<sup>2</sup>, Camellia W. Adams<sup>4</sup>, Leonard G. Presta<sup>5</sup>, and Paul Carter<sup>1\*</sup>

Departments of <sup>1</sup>Molecular Oncology, <sup>2</sup>Molecular Biology, <sup>3</sup>Antibody Technologies, and <sup>4</sup>Immunology, Genentech Inc., South San Francisco, CA 94080. <sup>5</sup>Current address: Department of Molecular and Cell Biology, ImClone Systems, Inc., New York, NY 10014. \*Corresponding author (e-mail: [pjc@gene.com](mailto:pjc@gene.com)).

Received 2 March 1998; accepted 4 June 1998

**Production of bispecific IgG (BsIgG) by coexpressing two different antibodies is inefficient due to unwanted pairings of the component heavy and light chains. To overcome this problem, heavy chains were remodeled for heterodimerization using engineered disulfide bonds in combination with previously identified "knobs-into-holes" mutations. One of the variants, S354C:T366W/Y349'C:T366'S:L368'A:Y407'V, gave near quantitative (~95%) heterodimerization. Light chain mispairing was circumvented by using an identical light chain for each arm of the BsIgG. Antibodies with identical light chains that bind to different antigens were identified from an scFv phage library with a very restricted light chain repertoire for the majority (50/55) of antigen pairs tested. A BsIgG capable of simultaneously binding to the human receptors HER3 and cMpl was prepared by coexpressing the common light chain and corresponding remodeled heavy chains followed by protein A chromatography. The engineered heavy chains retain their ability to support antibody-dependent cell-mediated cytotoxicity as demonstrated with an anti-HER2 antibody.**

Keywords: applied immunology, antibody engineering, phage display

The therapeutic potential of bispecific antibodies has been minimally explored due to inefficient production methods. Robust recombinant routes have been developed for bispecific antibody fragments<sup>1-3</sup> but not for bispecific IgG (BsIgG). Many applications of bispecific antibodies will require an IgG as this format provides an Fc region, comprising C<sub>H</sub>2 and C<sub>H</sub>3 domains, that can confer long serum half-life and support secondary immune functions.

The method of choice for preparing BsIgG has been the coexpression of two different IgGs in hybrid hybridomas<sup>4</sup>. IgG coexpression may produce up to 10 heavy (H) and light (L) chain pairings<sup>5</sup>, thereby compromising the yield of BsIgG and often imposing major purification challenges. IgG H chains form homodimers, as well as the desired heterodimers, and L chains are prone to pair with noncognate as well as their cognate H chains.

We describe a method for the construction of human BsIgG that eliminates L chain mispairing and greatly diminishes H chain mispairing (Fig. 1). Antibody H chains have previously been remodeled for heterodimerization by rational design<sup>6</sup> and optimization by phage<sup>7</sup>. We have investigated the ability of engineered inter-C<sub>H</sub>3 domain disulfide bonds to further enhance H chain heterodimerization. Disulfide bonds were also evaluated as an analytical tool to distinguish heterodimers from homodimers by SDS-PAGE as engineered disulfide bonds can increase the electrophoretic mobility of proteins when analyzed by SDS-PAGE under nonreducing conditions<sup>8-10</sup>. The L chain mispairing problem was circumvented by constructing a BsIgG from antibodies that use identical L chains. Such antibodies are routinely isolated from phage libraries that have extensive H chain repertoires and have unique<sup>11</sup> or very few<sup>12</sup> L chain sequences.

## Results

**Design of C<sub>H</sub>3 variants.** Antibody H chains were remodeled for heterodimerization using engineered disulfide bonds both on their own and in combination with sterically complementary, knobs-into-holes mutations<sup>6,7</sup>. Three criteria were used to identify suitable pairs of C<sub>H</sub>3 residues for creating an inter-H chain disulfide bond. First, the  $\alpha$ -carbons should be separated by distances similar to those found in natu-

rally occurring disulfide bonds (5.0–6.8 Å)<sup>13</sup>. Distances up to 7.6 Å were considered to allow for possible movement of the main chain. Second, the residue pairs should involve a different residue on each C<sub>H</sub>3 domain. Third, cysteine replacement of the residues should permit the formation of a disulfide bond with favorable geometry<sup>14</sup>.

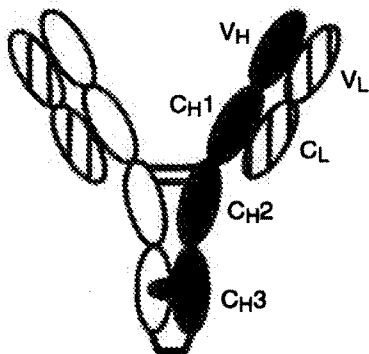
Six pairs of C<sub>H</sub>3 residues satisfy the criteria above and were chosen for cysteine replacement mutations. These six residue pairs are located near the edge of the C<sub>H</sub>3 domain interface either proximal (K392C with D399'C and V397C with T394'C) or distal (S354C with L351'C, and S354C, E356C, or E357C with Y349'C) to the C<sub>H</sub>2 domain (Fig. 2). In contrast, previously identified knobs-into-holes mutations (T366W/T366'S:L368'A:Y407'V) that enhance heterodimerization<sup>7</sup> are located at the center of the C<sub>H</sub>3 domain interface, away from the sites targeted for cysteine replacements (Fig. 2). These two engineering strategies were combined in an effort to further enhance H chain heterodimerization.

The cysteine replacement, L351C, was predicted and subsequently found to form disulfide-bonded homodimers as well as heterodimers, thus decreasing its utility. The cysteine mutant pair, V397C/T394'C, led to inefficient disulfide bond formation. The four remaining pairs of cysteine mutants (K392C/D399'C, S354C/Y349'C, E356C/Y349'C, and E357C/Y349'C) were installed in both possible orientations into the phage-optimized knobs-into-holes variant, T366W/T366'S:L368'A:Y407'V (ref. 7).

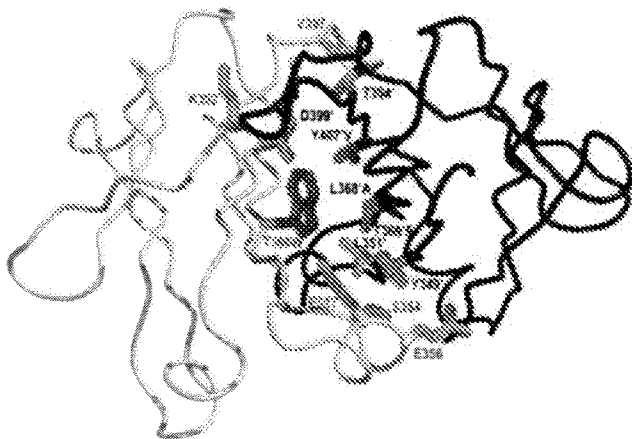
**Enhancing Fc heterodimerization.** C<sub>H</sub>3 variants were compared with wild-type in their ability to direct the formation of an anti-CD3/CD4-IgG antibody/immunoadhesin hybrid (Ab/IA)<sup>15</sup>. The CD4-IgG and anti-CD3 H chain variants were transiently coexpressed in human embryonic kidney 293 cells, along with the anti-CD3 L chain. The yield of Ab/IA was optimized by varying the ratios of transfected H chain to IA DNA as described previously<sup>6</sup>. The products were affinity-purified using staphylococcal protein A and quantified by SDS-PAGE and scanning laser densitometry<sup>6,7</sup>. Fc heterodimerization is evident from the molecular weight of the resultant Ab/IA hybrid as this is distinct from both IgG and IA homodimer. All Ab/IA variants (v1–v16) were recovered in yields similar to molecules containing the wild-type C<sub>H</sub>3 domain.

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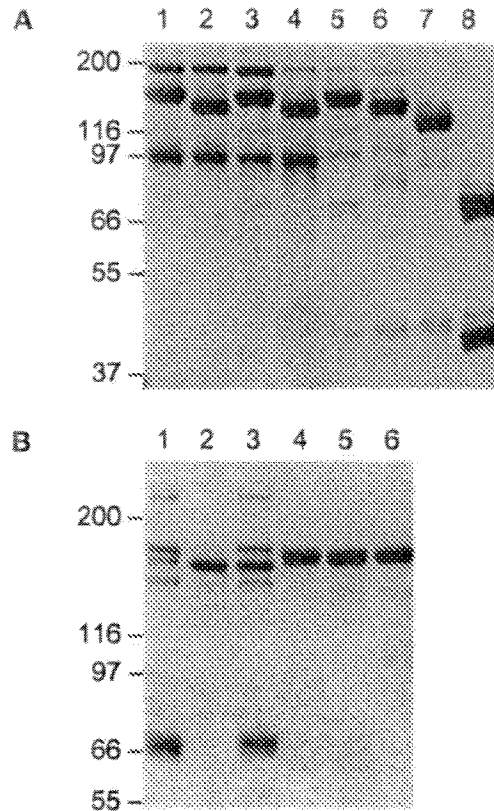
As with the parent molecules<sup>6,7</sup>, each of the six double cysteine replacement mutants (disulfide-C<sub>H3</sub> variants) gave rise to three major products: IgG, Ab/IA hybrid, and IA homodimer (Fig. 3A). However, Ab/IA hybrids from the disulfide-C<sub>H3</sub> variants show increased electrophoretic mobility compared with the Ab/IA constructed using wild-type C<sub>H3</sub> domains, consistent with formation of the designed disulfide bonds (Fig. 3A). Mutagenesis of the hinge cysteines to serine provided direct evidence for formation of the engineered disulfide bond. Covalently bonded Ab/IA hybrids were observed by SDS-PAGE for disulfide-C<sub>H3</sub> variant v11 in which the two hinge cysteines were mutated to serine. In contrast, such covalently bonded hybrids were not observed for molecules with hinge mutations and wild-type C<sub>H3</sub> domains (Fig. 3A). The disulfide variant, v1 (K392C/D399C), increased the Ab/IA yield over wild-type: 73% versus 51%, respectively (Table 1), whereas the five other disulfide variants constructed (v3 to v7) did not significantly impact Fc heterodimerization. This improvement with variant v1 apparently reflects disulfide bond formation rather than replacement of residues K392 and D399 as the corresponding double serine mutant (K392S/D399S, v2) gave Ab/IA yields similar to wild-type (Table 1).



**Figure 1.** Generic route to bispecific IgG. H chains (filled and unfilled) were remodeled so that they heterodimerize but do not homodimerize using knobs-into-holes C<sub>H3</sub> mutations<sup>6,7</sup> and an engineered disulfide bond (kinked line) between the C<sub>3</sub> domains. Antibodies that share the same L chain (striped) were chosen to circumvent the problem of L chains pairing with noncognate H chains. Two naturally occurring hinge region disulfide bonds are indicated by horizontal lines.



**Figure 2.** C<sub>3</sub> residues targeted for cysteine replacement mutations (cyan) highlighted in the 2.9 Å structure of human IgG, Fc (ref. 18). The knobs-into-holes mutations, T366W/T366S:L368A:Y407V (refs. 6 and 7; magenta) were introduced by molecular modeling.



**Figure 3.** SDS-PAGE analysis of protein A purified products from transiently coexpressed antibody H and L chains and IA. (A) Ab/IA production. Lane 1: wild-type; lane 2: v3; lane 3: v2; lane 4: v1; lane 5: v8; lane 6: v11; lanes 7 and 8: C231S:C234S/C231'S:C234'S hinge mutations in context of v11 and wild-type, respectively. (B) Bispecific IgG production. Lane 1: H<sub>1</sub> + L<sub>com</sub>; lane 2: H<sub>1</sub> + H<sub>mut</sub> + L<sub>com</sub>; lane 3: H<sub>1</sub> + L<sub>mut</sub>; lane 4: H<sub>mut</sub> + L<sub>com</sub>; lane 5: H<sub>mut</sub> + H<sub>mut</sub> + L<sub>com</sub>; and lane 6: H<sub>mut</sub> + L<sub>mut</sub>, where H<sub>1</sub> and H<sub>mut</sub> correspond to mutant (S354C:T366W) and wild-type versions of the anti-HER3 H chain, H<sub>1</sub> and H<sub>mut</sub> correspond to mutant (Y349C:T366S:L368A:Y407V) and wild-type versions of the anti-Mpl H chain, and L<sub>com</sub> represents the common L chain.

**Table 1.** Yields of Ab/IA hybrids from C<sub>H3</sub> variants.

Variant	Mutations		Yield of Ab/IA hybrid (%)
	Ab	IA	
wild-type	none	none	51 ± 1
v1	D399C	K392C	73 ± 3
v2	D399S	K392S	55 ± 1
v3	Y349C	S354C	54 ± 4
v4	Y349C	E356C	55 ± 6
v5	Y349C	E357C	57 ± 4
v6	L351C	S354C	56 ± 3
v7	T394C	V397C	57 ± 2
v8	T366W	T366S:L368A:Y407V	86.7 ± 2.3
v9	T366W:D399C	T366S:L368A:K392C:Y407V	86.5 ± 0.5
v10	T366W:K392C	T366S:D399C:L368A:Y407V	92 ± 1
v11	S354C:T366W	Y349C:T366S:L368A:Y407V	95 ± 2
v12	Y349C:T366W	S354C:T366S:L368A:Y407V	90 ± 1
v13	E356C:T366W	Y349C:T366S:L368A:Y407V	94 ± 2
v14	Y349C:T366W	E356C:T366S:L368A:Y407V	95.5 ± 0.5
v15	E357C:T366W	Y349C:T366S:L368A:Y407V	93 ± 2
v16	Y349C:T366W	E357C:T366S:L368A:Y407V	91.0 ± 1.0

The yield of Ab/IA was estimated by SDS-PAGE followed by scanning laser densitometry. Data are the mean (± SD) from two or more independent experiments. Mutations are denoted by the amino acid residue and number (Eu numbering scheme of Kabat et al.<sup>19</sup>) followed by the replacement amino acid. Multiple mutations are represented by the single mutations separated by a colon.



When evaluated in the context of the phage-optimized  $C_{n3}$  variant, v8, the cysteine replacement mutations increased the Ab/IA yield up to a maximum of approximately 95% (v11, v13, v14) or left it unchanged (v9) (Table 1). The engineered disulfide bonds exhibit a context-dependent effect upon Fc heterodimerization. For example, the mutations K392C/D399C are most effective in increasing the Ab/IA yield for an otherwise wild-type  $C_{n3}$ . In contrast, mutations S354C/Y349C and Y349C/E356C give the greatest improvement in Ab/IA yield for variant v8. One of the most successful variants, v11 (S354C:T366W/Y349C:T366S:L368A:Y407V), was utilized in the construction of a BsIgG.

**Identification of antibodies using identical L chains.** Panning of a large human scFv library<sup>12</sup> identified a panel of 11 antibodies specific for the extracellular domain (ECD) of human epidermal growth factor receptor 3 (HER3) also known as *c-erbB-3* (ref. 15). The  $V_H$  and  $V_L$  amino acid sequences of the anti-HER3 scFv were compared with 23 scFv that bind to the human thrombopoietin receptor, c-Mpl (data not shown). Five of the 11 anti-HER3 clones share an identical  $V_L$  amino acid sequence with one or more Mpl-binding clones. Conversely, seven out of 23 anti-Mpl scFv share the same  $V_L$  as one of the anti-HER3 clones. In contrast to the  $V_L$  sequences, the  $V_H$  amino acid sequences share an identity level of only 40–90% for any pair of anti-Mpl and anti-HER3 clones.

The frequency of identical L chains of clones derived by panning the phage library of Vaughan et al.<sup>12</sup> was assessed by comparing 117  $V_L$  amino acid sequences for scFv binding 11 different anti-

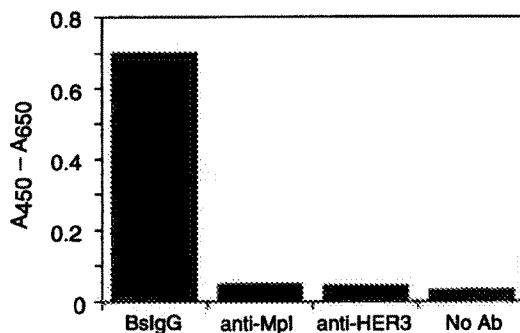
gens (Table 2). scFv sharing identical L chains were identified for the majority (50/55) of the possible pairwise combinations of two different antigen specificities. Identical L chains were found in all cases except where five or fewer sequences were available for one or both of the antigen specificities. This high frequency of antibodies sharing the identical L chain reflects the very limited size of the L chain repertoire in the phage library. The majority (95/117) of the  $V_L$  sequences are represented by just nine clads, each comprising sequences that differ by five amino acid residues or fewer from the consensus sequence of the clad. Our  $V_L$  sequence data closely match those reported by Vaughan et al.<sup>12</sup>

**Efficient construction of bispecific IgG.** The anti-Mpl scFv, 12B5 (Genbank accession number, AF048775), and anti-HER3 scFv clone H6 (Genbank accession number, AF048774) use identical  $V_L$  sequences and substantially different  $V_H$  sequences. These scFv fragments were reformatted as IgG to test the scheme for constructing BsIgG (Fig. 1). The common L chain was cotransfected with the two H chains containing the  $C_{n3}$  mutations from variant v11. The IgG products were then purified by protein A affinity chromatography and analyzed by SDS-PAGE (Fig. 3B).

The BsIgG preparation gave rise to a single major band showing greater mobility than IgG containing wild-type  $C_{n3}$  domains (Fig. 3B). This increase in electrophoretic mobility is consistent with the formation of the engineered disulfide bond in the BsIgG. As anticipated, the BsIgG, but not the parental anti-Mpl and anti-HER3 IgG, showed simultaneous binding to Mpl and HER3 ECD antigens in a sandwich ELISA (Fig. 4).

**Antibody with  $C_{n3}$  mutations supports efficient antibody-dependent cell-mediated cytotoxicity.** We also investigated the effect of Fc mutations (S354C:T366W and Y349C:T366S:L368A:Y407V) on the ability of an IgG to support antibody-dependent cell-mediated cytotoxicity (ADCC). We chose to make this comparison in the context of a monospecific, humanized anti-HER2 IgG, huMAb4D5-5 (ref. 16), rather than the BsIgG, because the control molecule containing a wild-type Fc region is easily prepared for the monospecific IgG but not for the BsIgG.

Anti-HER2 antibodies containing remodeled or wild-type Fc regions had similar potency in ADCC with the HER2-overexpressing breast cancer cell line SK-BR-3 (Fig. 5). Both antibodies showed comparable, low activity against the nontumor breast epithelial cell line HBL100, which expresses 33-fold less HER2 than SK-BR-3 cells<sup>17</sup> (Fig. 5). This maintenance of ADCC function is consistent with the prediction that the  $C_{n3}$  mutations are fully buried and do not propagate major structural changes to the surface of the corresponding  $C_{n3}$  domains.



**Figure 4.** Sandwich ELISA for detection of simultaneous binding to Mpl-IgG and HER3-IgG. Antibodies tested were the anti-Mpl x anti-HER3 BsIgG containing the mutations Y349C:T366S:L368A:Y407V/T366W:S354C, and the corresponding parental anti-Mpl or anti-HER3 antibodies with mutated Fc regions.

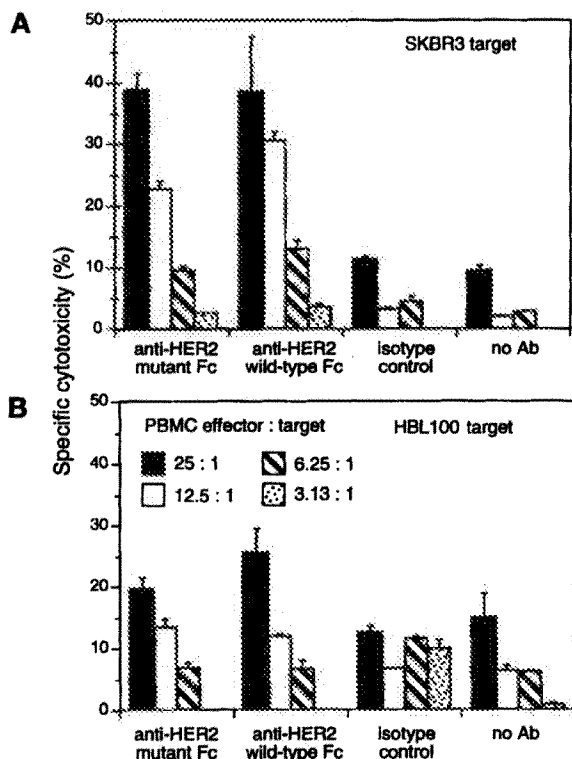
**Table 2.** Usage of identical  $V_L$  by scFv binding to different target antigens.

First specificity	Total # scFv	Second specificity										
		Axl	GCSF-R	IgE	IgE-R	c-Mpl	MuSK	NpoR	Rse	HER3	Ob-R	VEGF
Axl	12	2	2	0	1	2	1	0	3	2	2	1
GCSF-R	11	2	0	1	2	2	1	2	2	2	3	2
IgE	2	0	1	0	1	1	0	1	1	1	1	0
IgE-R	4	1	2	1	0	1	0	1	2	1	1	1
c-Mpl	23	3	3	1	1	5	5	3	5	7	5	2
MuSK <sup>a</sup>	3	1	1	0	0	3	0	1	1	2	1	1
NpoR	5	0	2	1	1	2	1	0	1	2	2	1
Rse	20	5	3	1	3	8	2	1	7	7	5	2
HER3	11	2	2	1	1	5	2	2	4	3	4	4
Ob-R	18	5	3	1	1	9	1	2	8	9	7	1
VEGF	8	1	3	0	1	2	2	2	1	4	2	2

Values are the frequencies of scFv sharing identical  $V_L$  amino acid sequences identified by alignment of 117  $V_L$  sequences for clones obtained using the library of Vaughan et al.<sup>12</sup> Axl: Axl receptor tyrosine kinase; GCSF-R: granulocyte colony stimulating factor receptor; IgE-R: IgE receptor; c-Mpl: human thrombopoietin receptor; MuSK: muscle-specific tyrosine kinase receptor; NpoR: Npo receptor; Rse: Rse receptor tyrosine kinase; HER3: human epidermal growth factor receptor 3; Ob-R: leptin receptor; VEGF: vascular endothelial growth factor. All antigens are human in origin except for IgE, which is murine.



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**Figure 5.** ADCC mediated by huMAB4D5-5 (Ref. 16) containing either a mutant (S354C:T366W/Y349C:T366S:L368A:Y407V) or wild-type Fc region or an isotype-matched control antibody (E25, Ref. 35) and human peripheral blood mononuclear cell (PBMC) effector cells. Data are the mean of triplicate measurements.

## Discussion

We have developed an efficient method for the construction of human BsIgG by remodeling antibody H chains for heterodimerization and using a single L chain to circumvent L chain mispairing (Fig. 1). We anticipate that BsIgG technology will be applicable to antibodies recognizing virtually any pair of antigens, as antibodies with different antigen-binding specificities that share identical L chains have been routinely identified by us (Table 1) and others<sup>11,12</sup>. In addition, the C<sub>H</sub>3 mutations required for H chain heterodimerization can be incorporated into H chains of any antigen-binding specificity. These engineered H chains promise to be useful in heterodimerization of other human immunoglobulins because the mutated residues are fully conserved across human IgG isotypes and the majority of the C<sub>H</sub>3 interface residues are highly conserved<sup>18-20</sup>. The engineered H chain variants may also be applied directly to the construction of bispecific immunoadhesins<sup>21</sup> in addition to the BsIgG and Ab/IA shown here.

The BsIgG comigrates on SDS-PAGE with one of the five products from either parental IgG (Fig. 3B). The electrophoretic mobilities of the minor contaminants in the BsIgG preparation are consistent with the presence of antibody aggregates and monomers, and three lines of evidence taken together support the notion that our BsIgG preparation contains little contaminating parental IgG. First, the BsIgG contains an Fc region identical to that of variant v11, which yielded 95% of the Ab/IA hybrid. It seems very likely that this same Fc region would be equally effective in directing the heterodimerization of two antibody H chains. Second, contamination of the BsIgG with parental IgG would be expected to give rise to all five of the parental bands rather than just one. Third, the BsIgG preparation binds simultaneously to

both antigens, whereas each parental IgG binds only to its respective, cognate antigen.

The potential risk of eliciting an antibody or T cell response is a frequently voiced concern when engineering proteins for human therapy. These immunogenicity issues can only be definitively addressed through clinical trials. Nevertheless, the risk of immunogenicity seems small as the C<sub>H</sub>3 mutations are fully buried and few in number (six). Humanized antibodies containing several dozen foreign and mainly exposed residues in their variable domains have elicited undetectable<sup>22-25</sup> or only minor<sup>26,27</sup> antiglobulin responses.

Antigen-binding affinities in the low nanomolar or even subnanomolar range are desirable and may be necessary for each arm of a BsIgG destined for human therapy. In some cases it will likely be possible to construct high-affinity human BsIgG using scFv isolated directly from a large naive phage library. Vaughan et al.<sup>12</sup> have reported many high-affinity scFv ( $K_D=0.3-8.0$  nM) from the same scFv phage library we used in this study to identify antibodies using the same L chains. These high-affinity scFv include several with specificities that have obvious clinical relevance; tumor-associated antigen (CEA), a radionuclide chelator (DPTA), and a chemotherapeutic drug (doxorubicin). Thus, high-affinity antigen binding is possible even with a very limited L chain repertoire. These high affinities presumably reflect the fact that the major determinants contributing to the specificity and energetics of antigen binding reside on the H chain for these phage-derived antibodies.

Sometimes it may be necessary to increase the binding affinity for one or both of the chosen antigens for a BsIgG. For example, in the case of the BsIgG evaluated in this paper, the anti-Mpl binding affinity is low ( $K_D=96$  nM for Fab fragment, data not shown). Affinity maturation of antibody fragments from the nanomolar to picomolar range has been accomplished by mutagenesis of H and L chains and selection using phage display libraries<sup>28,29</sup>. For BsIgG, any mutation of the L chain would have to be evaluated in terms of maintaining binding to the two different antigens.

It may be desirable to construct a BsIgG using the common L chain format (Fig. 1) in conjunction with H and L chains derived from an existing antibody. In this case it will be necessary to identify a second H chain that pairs with the original L chain and binds the second antigen of interest. The Fab phage library and the panning strategy of Figini et al.<sup>30</sup> offer a promising route for identifying such an H chain.

## Experimental protocol

**Modeling of disulfide bonds.** Disulfide bonds were modeled into the human IgG, Fc<sup>3</sup> as described for huMAB4D5-8 Fv (ref. 31) using Insight II release 95.0 (Molecular Simulations, San Diego, CA).

**Construction of C<sub>H</sub>3 variants.** Mutations were introduced into the C<sub>H</sub>3 domain of a humanized anti-CD3 H chain or CD4-IgG by site-directed mutagenesis<sup>31</sup>. Mutants were verified by dideoxynucleotide sequencing<sup>31</sup> using Sequenase version 2.0 (United States Biochemicals, Cleveland, OH).

**Expression and purification of Ab/IA variants.** Human embryonic kidney 293S cells were cotransfected by the calcium phosphate method<sup>31</sup> with a total of 10 µg DNA encoding CD4-IgG plus anti-CD3 L and H chains. The ratio of phagemid DNA for L and H chains was fixed at 3:1 so that the L chain would not be limiting. Additionally, the IA-encoding DNA was used in excess to that for the H chain because the IA is less efficiently expressed than the IgG. The transfected DNA ratios tested ranged from 3:1:3 through 8:1:3 for IA:H chain:L chain. The 293S cells were washed with phosphate-buffered saline (PBS) prior to transfection to remove any residual immunoglobulin from the fetal calf serum used in the media. Following the transfection (72 h) Fc-containing proteins were purified from conditioned media using immobilized protein A (ProSep A, BioProcessing Ltd., Consett, UK) and buffer-exchanged into PBS. Iodoacetamide was added to protein preparations to a final concentration of 50 mM to prevent shuffling of disulfide bonds.

**Quantitation of Fc-containing proteins.** Samples were electrophoresed in 8% (Ab/IA) or 6% (BsIgG) polyacrylamide gels (Novex, San Diego, CA) and visualized by staining with Serva blue G (Serva, Heidelberg, Germany). Gels

were destained leaving a faint blue background to permit detection and quantitation of minor contaminants. Dried gels were analyzed by scanning laser densitometry (GS-670; Bio-Rad, Hercules, CA) and protein products were quantified with Molecular Analyst software (Bio-Rad).

**Identification of scFv by phage library panning.** scFv binding HER3 were obtained using a large human scFv phage library<sup>11</sup> (Cambridge Antibody Technology, Melbourne, UK) and panning for three rounds using HER3-IgG (10 µg in 1 ml PBS) coated Immuntubes (Maxisorp; Nunc, Naperville, IL). Panning and phage rescue were then performed as described by Vaughan et al.<sup>12</sup> with the following modifications. A humanized anti-IgE antibody<sup>13</sup> at a concentration of 1 mg/ml was included in each panning step to absorb Fc-binding phage. Clones from rounds two and three of panning were screened by phage and scFv ELISA using the HER3-IgG and also a control IA, CD4-IgG<sup>26</sup>. The diversity of antigen-positive clones was analyzed by PCR-amplification of the scFv insert using the primers, fdtseq and PUC19 reverse<sup>11</sup> and by digestion with BstNI<sup>14</sup> (ref. 37). Up to five clones per BstNI fingerprint were then cycle-sequenced using fluorescent dideoxy chain terminators (Applied Biosystems, Foster City, CA) using M13 reverse (New England Biolabs, Beverly, MA) and myc seq 10 primers<sup>11</sup>. Samples were analyzed using automated DNA sequencers (models 373 and 377; Applied Biosystems) and sequences analyzed using Sequencher (Gene Codes, Ann Arbor, MI).

**Alignment of V<sub>L</sub> sequences.** The nucleotide sequence data for scFv fragments were translated to derive corresponding protein sequences. The V<sub>L</sub> sequences were then compared using the program ALIGN with the algorithm of Feng and Doolittle<sup>38,40</sup> to calculate the percentage identity between all pairwise combinations of chains.

**ADCC.** Cytotoxicity assays were performed with <sup>51</sup>Cr-labeled SK-BR-3 and HBL100 target cells and human peripheral blood lymphocytes as effector cells as described<sup>17</sup>, except that the lymphocytes were not activated with IL-2. The concentration of antibodies in the ADCC assays was 125 ng/mL.

**ELISA.** PBS was used as buffer for all steps. Individual wells of a 96 well plate (Maxisorp, Nunc) were coated overnight with Mpl-IgG at 5 µg/ml, washed and then blocked for 1 h with 0.5% (wt/vol) bovine serum albumin. The primary antibodies were the anti-Mpl x anti-HER3 BslgG containing the mutations Y349C:T366S:L368A:Y407V/T366'W:S354'C and corresponding parental anti-Mpl or anti-HER3 antibodies with mutated Fc regions. The primary antibodies (1 µg/mL) were individually incubated for 2 h at 23°C with biotinylated HER3-IgG and a 1:5000 dilution of streptavidin-horseradish peroxidase conjugate (Boehringer Mannheim, Indianapolis, IN) and then added to the wells and incubated for an additional 1 h at 23°C. Peroxidase activity was detected with TMB reagents as directed by the vendor (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

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1. Holliger, P. and Winter, G. 1993. Engineering bispecific antibodies. *Current Opin. Biotechnol.* **4**:446-449.
2. Carter, P., Ridgway, J., and Zhu, Z. 1995. Toward the production of bispecific antibody fragments for clinical applications. *J. Hematother.* **4**:463-470.
3. Plückthun, A. and Pack, P. 1997. New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology* **3**:83-105.
4. Milstein, C. and Cuello, A.C. 1983. Hybrid hybridomas and their use in immunohistochemistry. *Nature* **305**:537-540.
5. Suresh, M.R., Cuello, A.C., and Milstein, C. 1986. Bispecific monoclonal antibodies from hybrid hybridomas. *Methods Enzymol.* **121**:210-228.
6. Ridgway, J.B.B., Presta, L.G., and Carter, P. 1996. "Knobs-into-holes" engineering of antibody C<sub>3</sub> domains for heavy chain heterodimerization. *Protein Eng.* **9**:617-621.
7. Atwell, S., Ridgway, J.B.B., Wells, J.A., and Carter, P. 1997. Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library. *J. Mol. Biol.* **270**:26-35.
8. Villafranca, J.E., Howell, E.E., Voet, D.H., Strobel, M.S., Ogden, R.C., Abelson, J.N., and Kraut, J. 1983. Directed mutagenesis of dihydrofolate reductase. *Science* **222**:782-788.
9. Perry, L.J. and Wetzel, R. 1984. Disulfide bond engineered into T4 lysozyme: stabilization of the protein toward thermal inactivation. *Science* **276**:555-557.
10. Wells, J.A. and Powers, D.P. 1986. In vivo formation and stability of engineered disulfide bonds in subtilisin. *J. Biol. Chem.* **261**:6564-6570.
11. Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D., and Winter, G. 1994. Antibody fragments from a "single pot" phage display

- library as immunochemical reagents. *EMBO J.* **13**:692-698.
12. Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J. et al. 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature Biotechnology* **14**:309-314.
13. Srinivasan, N., Sowdhamini, R., Ramakrishnan, C., and Balaram, P. 1990. Conformations of disulfide bridges in proteins. *Int. J. Peptides Protein Res.* **38**:147-155.
14. Chamow, S.M., Zhang, D.Z., Tan, X.Y., Mhatre, S.M., Marsters, S.A., Peers, D.H. et al. 1994. A humanized, bispecific immunoadhesin-antibody that retargets CD3<sup>+</sup> effectors to kill HIV-1-infected cells. *J. Immunol.* **153**:4268-4280.
15. Plowman, G.D., Whitney, G.S., Neubauer, M.G., Green, J.M., McDonald, V.L., Todaro, G.J., and Shoyab, M. 1990. Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc. Natl. Acad. Sci. USA* **87**:4905-4909.
16. Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B.B., Henner, D., Wong, W.L.T., et al. 1992. Humanization of an anti-p185<sup>HER2</sup> antibody for human cancer therapy. *Proc. Natl. Acad. Sci. USA* **89**:4285-4289.
17. Lewis, G.D., Figari, I., Fendly, B., Wong, W.L., Carter, P., Gorman, C., and Shepard, H. M. 1993. Differential responses of human tumor cells lines to anti-p185<sup>HER2</sup> monoclonal antibodies. *Cancer Immunol. Immunother.* **37**:255-263.
18. Deisenhofer, J. 1981. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8 Å resolution. *Biochemistry* **20**:2361-2370.
19. Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., and Foeller, C. (eds.). 1991. pp. 688-696 in *Sequences of proteins of immunological interest*, 5th ed., Vol. 1. NIH, Bethesda, MD.
20. Miller, S. 1990. Protein-protein recognition and the association of immunoglobulin constant domains. *J. Mol. Biol.* **216**:965-973.
21. Dietsch, M.T., Smith, V.F., Cosand, W.L., Damle, N.K., Ledbetter, J.A., Linsley, P.S., and Aruffo, A. 1993. Bispecific receptor globulins, novel tools for the study of cellular interactions. Preparation and characterization of an E-selectin/P-selectin bispecific receptor globulin. *J. Immunol. Methods* **162**:123-132.
22. Hale, G., Dyer, M.J.S., Clark, M.R., Phillips, J.M., Marcus, R., Fiechmann, L. et al. 1988. Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet* **2**:1394-1399.
23. Caron, P.C., Juric, J.G., Scott, A.M., Finn, R.D., Divigi, R.D., Graham, M.C. et al. 1994. A phase 1B trial of humanized monoclonal antibody M195 (anti-CD33) in myeloid leukemia: specific targeting without immunogenicity. *Blood* **83**:1760-1768.
24. Sharkey, R.M., Malik, J., Shevitz, J., Behr, T., Dunn, R., Swayne, L.C. et al. 1995. Evaluation of a complementarity-determining region-grafted (humanized) anti-carcinoma embryonic antigen monoclonal antibody in preclinical and clinical studies. *Cancer Res.* **55**:5935s-5945s.
25. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C.C., Dantis, L. et al. 1996. Phase II study of weekly intravenous recombinant humanized anti-p185<sup>HER2</sup> monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J. Clin. Oncol.* **14**:737-744.
26. Isaacs, J.D., Watts, R.A., Hazleman, B.L., Hale, G., Keogan, M.T., Cobbold, S.P., and Waldmann, H. 1992. Humanised monoclonal antibody therapy for rheumatoid arthritis. *Lancet* **340**:748-752.
27. Vincenti, F., Lantz, M., Birnbaum, J., Garovoy, M., Mould, D., Hakimi, J. et al. 1994. A phase I trial of humanized anti-interleukin 2 receptor antibody in renal transplantation. *Transplantation* **63**:33-38.
28. Yang, W.-P., Green, K., Pinz-Sweeney, S., Briones, A.T., Burton, D.R. and Barbas, C.F. 1995. CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. *J. Mol. Biol.* **254**:392-403.
29. Schlier, R., McCall, A., Adam, G.P., Marshall, K.W., Merritt, H., Yim M. et al. 1996. Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J. Mol. Biol.* **263**:551-567.
30. Figini, M., Marks, J.D., Winter, G. and Griffiths, A.D. 1994. In vitro assembly of repertoires of antibody chains on the surface of phage by renaturation. *J. Mol. Biol.* **239**:68-78.
31. Rodrigues, M.L., Presta, L.G., Kotts, C.E., Wirth, C., Mordenti, J., Osaka, G. et al. 1995. Development of a humanized disulfide-stabilized anti-p185<sup>HER2</sup> Fv-β-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug. *Cancer Res.* **55**:63-70.
32. Kunkel, T.A., Roberts, J.D., and Zakour, R.A. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367-382.
33. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
34. Gorman, C.M., Gies, D.R., and McCray, G. 1990. Transient production of proteins using an adenovirus transformed cell line. *DNA and Protein Engineering Techniques* **2**:3-10.
35. Presta, L.G., Lahr, S.J., Shields, R.L., Porter, J.P., Gorman, C.M., Fendly, B.M., and Jardieu, P. M. 1993. Humanization of an antibody directed against IgE. *J. Immunol.* **151**:2623-2632.
36. Byrn, R.A., Mordenti, J., Lucas, C., Smith, D., Marsters, S.A., Johnson, J.S. et al. 1990. Biological properties of a CD4 immunoadhesin. *Nature* **344**:667-670.
37. Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., and Winter, G. 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* **222**:581-597.
38. Feng, D.F. and Doolittle, R.F. 1985. Aligning amino acid sequences: comparison of commonly used methods. *J. Mol. Evol.* **21**:112-123.
39. Feng, D. F. and Doolittle, R. F. 1987. Progressive alignment as a prerequisite to correct phylogenetic trees. *J. Mol. Evol.* **25**:351-360.
40. Feng, D.F. and Doolittle, R.F. 1990. Progressive alignment and phylogenetic tree construction of protein sequences. *Methods Enzymol.* **183**:375-387.
41. Xie, M.H., Yuan, J., Adams, C., and Gurney, A. 1997. Direct demonstration of MuSK involvement in acetylcholine receptor clustering through identification of agonist ScFv. *Nature Biotechnology* **15**:768-771.