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(57) Abstract: The present invention relates to methods for the manufacture in transgenic non-human mammals of a diverse repertoire of functional, affinity-matured homodimeric VH binding complexes in response to antigen challenge and uses thereof, especially the derivation of antigen specific VH binding domains. In particular the methods of the present invention involve the step of providing one or more heterologous VH binding complex loci in transgenic non-human mammal, wherein each VH binding complex locus is defined by the formula 5'-A-B-C-D-3' wherein: A comprises one or more V gene segments, one or more D gene segments and one or more J gene segments; B is optional and encodes one or more characterised binding domains of known specificity; C encodes a CH2-CH3 dimerisation domain; and D encodes one or more characterised binding domains of known specificity. The homodimeric VH binding complexes are minimally tetravalent and bispecific but may also be multivalent and bispecific or multivalent and multispecific.



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Germ-line manipulation 1

FIELD OF THE INVENTION

The present invention relates to methods for the manufacture in transgenic non-human mammals of a diverse repertoire of functional, affinity-matured homodimeric VH binding
5 complexes in response to antigen challenge and uses thereof, especially the derivation of antigen specific VH binding domains. The homodimeric VH binding complexes are minimally tetravalent and bispecific but may also be multivalent and bispecific or multivalent and multispecific.

10 In the following description, all amino acid residue position numbers in VH domains are given according to the numbering system devised by Kabat (Kabat, E., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) United States Public Health Services Publication No. 91-3242, National Institutes of Health, Bethesda, MD).

BACKGROUND TO THE INVENTION

Antibodies

15 The structure of antibodies is well known in the art. Most natural antibodies are tetrameric, comprising two heavy chains and two light chains. The heavy chains are joined to each other via disulphide bonds between hinge domains located approximately half way along each heavy chain. A light chain is associated with each heavy chain on the N-terminal side of the hinge domain. Each light chain is normally bound to its respective heavy chain by a
20 disulphide bond close to the hinge domain.

When an antibody molecule is correctly folded, each chain folds into a number of distinct globular domains joined by more linear polypeptide sequences. For example, the light chain folds into a variable (VL) and a constant (CL) domain. Heavy chains have a single variable domain VH, a first constant domain (CH1), a hinge domain and two or three further constant
25 domains. The heavy chain constant domains and the hinge domain together form what is generally known as the constant region of an antibody heavy chain. The effector functions of natural antibodies are provided by the heavy chain constant region.

Interaction of the heavy (VH) and light (VL) chain variable domains results in the formation of an antigen binding region (Fv). Interaction of the heavy and light chains is facilitated by the CH1 domain of the heavy chain and the C κ or C λ domain of the light chain. Generally, both VH and VL are required for antigen binding, although heavy chain dimers and amino-terminal fragments thereof have been shown to retain activity in the absence of light chain (Jaton et al. (1968) Biochemistry, 7, 4185-4195).

Within the variable domains of both heavy (VH) and light (VL) chains, some short polypeptide segments show exceptional variability. These segments are termed hypervariable regions or complementarity determining regions (CDRs). The intervening segments are called framework regions (FRs). In each of the VH and VL domains, there are three CDRs (CDR1-CDR3).

In mammals, there are five classes of antibody: IgA, IgD, IgE, IgG and IgM, with four IgG and two IgA subtypes present in humans.

Class	H chain	L chain	Subunits	mg/ml	Notes
IgG	gamma	kappa or lambda	H2L2	6-13	transferred across placenta
IgM	mu	kappa or lambda	(H2L2)5	0.5-3	first antibodies to appear after immunization
IgA	alpha	kappa or lambda	(H2L2)2	0.6-3	much higher concentrations in secretions
IgD	delta	kappa or lambda	H2L2	<0.14	function uncertain
IgE	epsilon	kappa or lambda	H2L2	<0.0004	binds to <u>basophils</u> and mast cells sensitizing them for certain <u>allergic reactions</u>

Antibody classes differ in their physiological function. For example, IgG plays a dominant role in a mature immune response. IgG (in its four isotypes) provides the majority of antibody-based immunity against invading pathogens.

5 IgM is involved in complement fixing and agglutination. IgM is expressed on the surface of B cells and also in a secreted form with very high affinity for eliminating pathogens in the early stages of B cell mediated immunity (i.e. before there is sufficient IgG to eliminate the pathogens).

10 IgA is the major class of antibody in secretions – tears, saliva, colostrum, mucus – and thus plays a role in local immunity. IgA can be found in areas containing mucus (e.g. in the gut, in the respiratory tract or in the urinogenital tract) and prevents the colonization of mucosal areas by pathogens.

IgD functions mainly as an antigen receptor on B cells.

IgE binds to allergens and triggers histamine release from mast cells (the underlying mechanism of allergy) and also provides protection against helminths (worms).

15 Normal human B cells contain a single heavy chain locus on chromosome 14 from which the gene encoding a heavy chain is produced by rearrangement. In the mouse, the heavy chain locus is located on chromosome 12. A normal heavy chain locus comprises a plurality of V gene segments, a number of D gene segments and a number of J gene segments. There are over 50 human V gene segments present in the human genome, of which only 39 are
20 functional.

Most of a VH domain is encoded by a V gene segment, but the C terminal end of each VH domain is encoded by a D gene segment and a J gene segment. VDJ rearrangement in B cells, followed by affinity maturation, provides each VH domain with its antigen binding specificity. Sequence analysis of normal H2L2 tetramers demonstrates that diversity results
25 primarily from a combination of VDJ rearrangement and somatic hypermutation (Xu and Davies, (2000) Immunity, 13, 37-45). Somatic hypermutation during B-cell activation is a function of the AID enzyme (for review, see Di Noia, J. M. and Neuberger, M. S. (2007) Ann. Rev. Biochem., 76, 1-22).

Fully human antibodies (H2L2) can now be derived from transgenic mice in response to antigen challenge. Such transgenic mice comprise a single human heavy chain locus and a separate light chain locus. The comparable mouse heavy and light chain loci are deleted or suppressed so that only human antibodies are produced in the absence of mouse antibodies

5 (Jakobovits A., The long-awaited magic bullets: therapeutic human monoclonal antibodies from transgenic mice, *Expert Opin. Investig. Drugs*, 1998 Apr; 7(4):607-14. Links; Davis C.G., Jia X.C., Feng X., Haak-Frendscho M., Production of human antibodies from transgenic mice, *Methods Mol. Biol.* (2004) 248:191-200; Kellermann S.A., Green L.L., Antibody discovery: the use of transgenic mice to generate human monoclonal antibodies for

10 therapeutics, *Curr. Opin. Biotechnol.*, 2002 Dec. 13 (6):593-7; EP1690935; US2005287630; WO9634096; WO9402602).

With the advent of new molecular biology techniques, the presence of heavy chain-only antibody (devoid of light chain) was identified in B cell proliferative disorders in man (heavy chain disease) and in murine model systems. Analysis of heavy chain disease at the

15 molecular level showed that mutations and deletions at the level of the genome could result in inappropriate expression of the heavy chain CH1 domain, giving rise to the expression of heavy chain-only antibody lacking the ability to bind light chain (Hendershot et al., (1987) *J. Cell Biol.*, 104, 761-767; Brandt et al., (1984) *Mol. Cell. Biol.*, 4, 1270-1277).

It has been shown that camelids, as a result of natural gene mutations, produce functional

20 IgG2 and IgG3 heavy chain-only dimers which are unable to bind light chain due to the absence of the CH1 domain, which mediates binding to the light chain (Hamers-Casterman et al., (1993) *Nature*, 363, 446-448). A characterising feature of the camelid heavy chain-only antibody is a particular subset of camelid V gene segments, which provides improved solubility relative to human and normal camelid V gene segments where the presence of the

25 VL confers solubility on the Fv antigen binding domain. The camelid VH domains derived from this particular V gene segment subset are usually referred to as VHH domains.

It has also been shown that species such as shark produce a heavy chain-only-like binding protein family, probably related to the mammalian T-cell receptor or antibody light chain (Stanfield et al., (2004) *Science*, 305, 1770-1773).

30 For the production of camelid heavy chain-only antibody, the heavy chain locus in the camelid germline comprises gene segments encoding some or all of the possible heavy chain

constant regions. During maturation, a re-arranged VDJ binding domain is spliced onto the 5' end of the gene segment encoding the hinge domain, to provide a re-arranged gene encoding a heavy chain which lacks a CH1 domain and is therefore unable to associate with a light chain.

Camelid VHH domains contain a number of characteristic amino acids at positions 37, 44, 45 and 47. These conserved amino acids are thought to be important for conferring solubility on heavy chain-only antibodies.

Heavy chain-only monoclonal antibodies can be recovered from B cells of camelid spleen by standard cloning technology or from B cell mRNA by phage or other display technology. Heavy chain-only antibodies derived from camelids are of high affinity. Sequence analysis of mRNA encoding heavy chain-only antibody demonstrates that diversity results primarily from a combination of VDJ rearrangement and somatic hypermutation as is also observed in the production of normal tetrameric antibodies (see Nguyen, V.K., Desmyter, A., Muyldermans, S., (2001) Adv. Immunol. 79, 261-296 and references therein).

An important feature of natural camelid VHH and some natural and engineered human VH binding domains is that each domain binds as a monomer with no dependency on dimerisation with a VL domain, for improved solubility.

Recently, methods for the production of soluble, non-camelid heavy chain-only antibodies in transgenic non-human mammals have been developed (see WO02/085945 and WO02/085944). Functional heavy chain-only antibody can be produced from transgenic non-human mammals (preferably mice) as a result of antigen challenge (Janssens, R., Dekker, S., Hendriks, R.W., Panayotou, G., van Remoortere, A., Kong-a San, J., Grosveld, F.G., and Drabek, D., Generation of heavy chain only antibodies in mice, PNAS (2006) online Oct 2 doi:10.1073/pnas0601108103 PNAS 2006, 103(41):15130-5). The heavy chain-only antibodies are derived as a result of antigen challenge, have high binding affinity as a result of antibody maturation in B cells, and can be selected using established hybridoma technology. Any class of antibody can be produced in the absence of light chain (e.g. IgG, IgA, IgD, IgE, IgM).

It has also been shown that the repertoire of antibody response can be greatly improved by increasing the number of heavy chain-only loci present in a transgenic non-human mammal used to produce class-specific, heavy chain-only antibodies since, where a transgenic non-human mammal possesses multiple heavy chain-only loci, these loci are subject to allelic

exclusion. Therefore, only one locus is stochastically chosen and recombined successfully, resulting in the production of a heavy chain-only antibody (see PCT/IB2007/001491). Multiple VH heavy chain loci can, therefore, be introduced into the same transgenic non-human mammal so as to maximise the antibody repertoire and diversity obtainable from the mammal. When antigenically challenged, the transgenic non-human mammal “selects” the locus comprising the V gene segment which is best suited to respond to the specific antigen challenge to the exclusion of the remaining loci.

Heavy chain-only antibodies that can be generated by the methods of the invention show high binding affinity as a result of the transgenic non-human mammal being able to “choose” from a range of loci, from which V, D and J gene segment rearrangements and somatic mutations can occur, generally in the absence of an enlarged CDR3 loop. Essentially normal B cell maturation is observed with high levels of heavy chain-only antibody present in isolated plasma (provided that the CH1 domain has been eliminated from all antibody classes present in the recombinant locus). B cell maturation and the secretion of assembled dimers (e.g. IgG) or multimers (e.g. IgM) has no dependency on the presence or expression of light chain genes.

Production of Antibody-based Products

The production of antibody-based products by genetic engineering, in particular the production of human or humanised antibody-based products, has resulted in the generation of new classes of medicines, diagnostics and reagents and, in parallel, opportunity for new industry, employment and wealth creation (see www.drugresearcher.com and www.leaddiscovery.co.uk). Antibody-based products will represent a high proportion of new medicines launched in the 21st century. Monoclonal antibody therapy is already accepted as a preferred route for the treatment for rheumatoid arthritis and Crohn’s disease and there is impressive progress in the treatment of cancer. Antibody-based products are also in development for the treatment of cardiovascular and infectious diseases. Most marketed antibody-based products recognise and bind a single, well-defined epitope on the target ligand (e.g. TNF α).

Antibody-based products are usually derived from natural tetrameric antibodies. There are many patents and applications which relate to the production of antibody-based products.

These patents and applications relate to routes of derivation (e.g. from transgenic mice), routes of manufacture and product-specific substances of matter. Such antibody-based

products vary from complete tetrameric antibodies through antibody fragments to engineered single chain Fv (scFv) molecules.

A number of groups have worked on the generation of heavy chain-only antibodies derived from natural tetrameric antibodies. Jaton et al., (1968) *Biochemistry*, 7, 4185-4195 and other
5 references cited therein describe the separation of the reduced heavy chain components of an affinity purified, well-characterised rabbit antibody, followed by the subsequent renaturation of the individual heavy chains. Immunological characterisation of the renatured heavy chains demonstrated that immunoglobulin heavy chain homodimers alone, free of light chain, are capable binding antigen.

10 Later Ward et al., (1989) *Nature*, 341, 544-546 demonstrated unambiguously that cloned murine VH domains, when expressed as soluble protein monomers in an *E. coli* expression system, retain the ability to bind antigen. Ward et al., (1989) *Nature*, 341, 544-546 also describe the isolation and characterisation of VH domains and set out the potential commercial advantages of this approach when compared with classic monoclonal antibody
15 production. Furthermore, it was suggested that the selection of VH domains with specific binding characteristics displayed on phage arrays could form the building blocks for engineered antibodies. It was also recognised that VH domains isolated from heavy chains which normally associate with a light chain, lack the solubility of the natural tetrameric antibodies. Hence Ward et al. used the term "sticky" to describe these molecules and
20 proposed that this "stickiness" can be addressed through the design of VH domains with improved solubility properties.

The improvement of human VH domain solubility has subsequently been addressed using combinations of randomized and site-directed approaches using phage display. For example, Davies and Riechmann (*Biotechnology* (1995) 13, 475-479) and others (see WO92/01047)
25 incorporated some of the features of VHH domains from camelid heavy chain-only antibodies in combination with phage display to improve human VH solubility whilst maintaining binding specificity.

Thus, human VH domains may be engineered for improved solubility characteristics (Davies and Riechmann, (1996) *Protein Eng.*, 9 (6), 531-537; Lutz and Muyldermans, (1999) *J. Immunol. Methods*, 231, 25-38) or solubility maybe be acquired by natural selection in vivo
30 (Tanha et al., (2001) *J. Biol. Chem.*, 276, 24774-24780). However, where VH binding

domains have been derived from phage libraries, intrinsic affinities for antigen remain in the low micromolar to high nanomolar range, in spite of the application of affinity improvement strategies involving, for example, affinity hot spot randomisation (Yau et al., (2005) *J. Immunol. Methods*, 297, 213-224), or affinity improvement using the AIDS enzyme (Iglesias-Ussel, M.D., Fan, M., Li, Z., Martin, A., Scharff, M.D., Forced expression of AID facilitates the isolation of class switch variants from hybridoma cells, *J. Immunol. Methods*, (2006) 316:59-66). Human VH produced by phage or alternative display technology lack the advantage of improved binding characteristics as a result of somatic mutations. Phage-derived antigen-specific human VH domains are laborious to select since they require many rounds of panning and subsequent mutagenesis in order to achieve high affinity binding characteristics. Camelid VHH domains require the same laborious procedure when isolated from phage or similar display libraries or require the immunization of large animals (llama or camels which also make classical antibodies) not amenable to established hybridoma technology. Moreover, camelid binding domains may prove antigenic and require humanization.

The production of heavy chain-only antibodies in transgenic non-human mammals from multiple loci (see WO02/085945, WO02/085944 and Janssens et al., (2006) *PNAS* online Oct 2 doi:10.1073/pnas.0601108103 *PNAS* 103(41):15130-5) as a result of antigen challenge overcomes many of these problems.

VH domains are also functional when configured at the amino or carboxyl termini of antibody complexes (see WO2006/008548 and PCT/GB2007/000258). Bi-specific antibody complexes are engineered immunoglobulin-based molecules capable of binding two different epitopes either on the same or different antigens. Bi-specific binding proteins incorporating antibodies alone or in combination with other binding agents show promise for treatment modalities where captured human immune functions elicit a therapeutic effect, for example the elimination of pathogens (Van Spriël et al., (1999) *J. Infect. Diseases*, 179, 661-669; Tacken et al., (2004) *J. Immunol.*, 172, 4934-4940; US 5,487,890), the treatment of cancer (Glennie and van der Winkel, (2003) *Drug Discovery Today*, 8, 503-5100) and immunotherapy (Van Spriël et al., (2000) *Immunol. Today*, 21, 391-397; Segal et al., (2001) *J. Immunol. Methods*, 248, 1-6; Lyden et al., (2001) *Nat. Med.*, 7, 1194-1201).

Manufacturing issues are compounded where a bi-specific antibody product is based on two or more H2L2 complexes. For example, co-expression of two or more sets of heavy and light

chain genes can result in the formation of up to 10 different combinations, only one of which is the desired heterodimer (Suresh et al., (1986) *Methods Enzymol.*, 121, 210-228).

To address this issue, a number of strategies have been developed for the production in mammalian cells of full length bi-specific IgG formats (BsIgG) which retain heavy chain effector function. BsIgGs require engineered “knob and hole” heavy chains to prevent heterodimer formation and utilise identical light chains to avoid light chain mispairing (Carter, (2001) *J. Immunol. Methods*, 248, 7-15). Alternative chemical cross-linking strategies have also been described for the production of complexes from antibody fragments each recognising different antigens (Ferguson et al., (1995) *Arthritis and Rheumatism*, 38, 190-200) or the cross-linking of other binding proteins, for example collectins, to antibody fragments (Tacke et al., (2004) *J. Immunol.*, 172, 4934-4940).

The development of diabodies or mini antibodies (BsAb) generally lacking heavy chain effector functions also overcomes heterodimer redundancy. These comprise minimal single chain antibodies incorporating VH and VL binding sites (scFv) which subsequently fold and dimerise to form a divalent bi-specific antibody monovalent to each of their target antigens (Holliger et al., (1993) *PNAS*, 90, 6444-6448; Muller et al., (1998) *FEBS Lett.*, 422, 259-264). In one instance, CH1 and light chain constant domains have been used as heterodimerisation domains for bi-specific mini antibody formation (Muller et al., (1998) *FEBS Lett.*, 259-264). A variety of recombinant methods based on *E. coli* expression systems have been developed for the production of BsAbs (Hudson, (1999) *Curr. Opin. Immunol.*, 11, 548-557), though it would appear that the cost and scale of production of clinical grade multivalent antibody material remains the primary impediment to clinical development (Segal et al., (2001) *J. Immunol. Methods*, 248, 1-6).

Recently, the BsAb concept has been extended to encompass di-diabodies, tetravalent bi-specific antibodies where the VH and VL domains on each H and L chain have been replaced by engineered pairs of scFv binding domains. Such constructs, whilst complex to engineer, can be assembled in mammalian cells in culture in the absence of heterodimer redundancy (Lu et al., (2003) *J. Immunol. Methods*, 279, 219-232).

scFvs have limitations due to inherent instability and folding inefficiency when produced and recovered from host cells, or when produced as intrabodies in a reducing intracellular environment (see der Maur et al., (2002) *J. Biol. Chem.*, 277, 45075-45085). In contrast VH

domains derived from heavy chain-only antibodies as typified by camelid VHH, show high thermodynamic stability relative to conventional antibody fragments (Dumoulin et al., (2002) Protein Science, 11, 500-515) and retain functional stability even in the presence of non-ionic and anionic surfactants, and harsh denaturing conditions such as urea (Dolk et al., (2005) Applied and Environmental Microbiology, 71, 442-450), important features for the recovery of functional antibody complexes in high yield from harsh manufacturing environments, and the maintenance of product structural and functional integrity both in vivo and in vitro. VHH and camelised or engineered VH binding domains also show the potential for greater target penetration of infectious agents than larger conventional antibody fragments (Stijlemans et al., (2004) J. Biol. Chem., 279, 1256-1261) and, when used as an "intrabody", retain intracellular structural and functional stability, blocking the production of porcine retrovirus by PK15 cells in culture (Dekker et al., (2003) J. Virol., 77, 12132-12139).

Camelid VHH binding domains are also characterised by a modified CDR3 loop. This CDR3 loop is not a feature of non-camelid antibodies, but in the camelid is considered to be a major influence on overall antigen affinity and specificity, so compensating for the absence of a VL domain in the camelid heavy chain-only antibody species (Desmyter et al., (1996) Nat. Struct. Biol., 3, 803-811; Riechmann and Muyldermans, (1999) J. Immunol. Methods, 23, 25-28).

An important and common feature of the VHH binding domains derived from camelid VHH heavy chain-only antibodies, camelised human VH heavy chain only antibodies, or VH binding domains from phage display libraries is that each binds as a monomer, with no dependency on dimerisation with a VL region for optimal solubility and binding affinity. These features appear particularly suited to the production of blocking agents and tissue penetration agents (for review see Holliger, P. & Hudson, P. J., (2005) Nature Biotechnology, 23, 1126-1136).

However, the benefits of VH binding domains found in heavy chain-only antibodies are only now being used to advantage in design of multimeric proteins as reagents, therapeutics and diagnostics (see WO2006/008548), although VH binding domains tethered by a natural antibody hinge region have previously been shown to retain binding characteristics within bispecific or bivalent constructs (Conrath et al., (2001) J. Biol. Chem., 276, 7346-7352).

The incorporation of multiple VH binding domains in combination with a dimerisation domain has clear advantage over parallel approaches using scFvs which must be engineered

from VH and VL domains with the associated potential of loss of specificity and avidity, the increased risk of antigenicity due to the presence of linker peptides, and inherent lack of stability relative to VH binding domains. VH binding domains derived using V gene segments from antibody-related gene families, such as T cell receptors or the shark immunoglobulin family, also provide alternatives to scFv for the generation of tetravalent bispecific, multivalent bispecific and multivalent multispecific VH binding molecules.

The presence of heavy chain CH2-CH3 constant domains provides the basis for the stable dimerisation seen in natural antibodies and provides recognition sites for post-translational glycosylation in addition to heavy chain effector functions. CH2-CH3 dimerisation domains have been used in the design of tetrameric monospecific homodimers or tetravalent bispecific homodimers carrying scFv binding domains at their amino and carboxyl termini (see Jendreyko et al. (2003) *J. Biol. Chem.*, 278, 47812-47819) or combinations of scFv binding domains and receptor binding proteins (Biburger et al. (2005) *J. Mol. Biol.*, 346, 1299-1311). CH2-CH3 domains have also been used to construct tetravalent bispecific homodimers using camelised VH and llama VHH binding domains derived from heavy chain-only antibodies (WO2006/008548).

The use of natural and engineered soluble VH domains for the construction of antibody complexes represents a significant improvement over available scFv binding technology and provides a robust route for the generation of antigen-specific, soluble and structurally stable VH monomers, and bivalent, tetravalent or multivalent VH binding complexes. Dimerisation domains may comprise natural or engineered immunoglobulin CH2-CH3 dimerisation domains including those lacking heavy chain effector function, for example the CH2-CH3 dimerisation domain derived from IgG4 (see Bruggemann, M. et al., *J. Exp. Med.*, (1987) 166, 1351-1361).

Thus soluble, affinity-matured VH binding domains derived from heavy chain-only antibodies following antigen challenge of transgenic animals comprising heavy chain-only immunoglobulin loci, as opposed to the "sticky" VH domains described by Ward et al., (loc. Cit.) or naïve VH domains derived from phage or similar display libraries, can form the basic building blocks for the subsequent production of soluble antigen-specific binding complexes and represent an important tool for the subsequent development of new therapeutics, diagnostics and reagents.

Selected phage derived VH binding domains require extensive work in vitro so as to derive high affinity soluble VH domains suited for therapy, whilst naturally soluble VHH domains derived from camelids must be humanised so as to reduce the likelihood of adverse immune responses if used for therapy in man. Even selected soluble antigen-specific high affinity VH domains must then be engineered back into antibody-based products. Thus selected, optimised soluble VH domains undergo yet further manipulation to generate dimeric, trimeric, tetrameric or multimeric configurations suited to specified therapeutic, diagnostic or reagent usage.

The optimisation or humanisation of VH binding domains and their subsequent engineering into soluble antibody complexes for use as therapeutics, diagnostics or reagents is scientifically testing and time consuming. There remains a need in the art for the improved derivation, selection and isolation of VH binding domains and VH binding complexes in vivo following antigen challenge. Furthermore, there remains a need in the art for affinity matured VH binding domains which can be produced in combination with selected characterised binding domains as a result of antigen challenge for use in diverse clinical, industrial and research applications.

THE INVENTION

The inventors have surprisingly established that homodimeric VH binding complexes can be assembled and secreted by B cells of non-human transgenic mammals in vivo as a result of antigen challenge. Characterised binding domains maybe engineered at both the amino and carboxyl termini of CH2-CH3 dimerisation domains of any class (IgM, IgG, IgD, IgA, IgE), so permitting the design, in vivo assembly and secretion of VH binding complexes comprising a novel antigen-specific, affinity-matured amino terminal VH binding domain resulting from antigen challenge, additionally characterised by binding domain(s) at the carboxy and optionally the amino terminus of the resulting homodimeric VH binding complex of the type shown in Figures 1 and 2. Homodimeric VH binding complexes of the invention may be tetravalent or multivalent, and either monospecific, bispecific or multispecific with respect to the epitopes recognised.

Accordingly, the invention provides a method for the production of a homodimeric VH binding complex in a transgenic non-human mammal in response to antigen challenge, comprising the step of providing one or more heterologous VH binding complex loci in that

mammal, wherein each VH binding complex locus is defined by the formula 5'-A-B-C-D-3' wherein: A comprises one or more V gene segments, one or more D gene segments and one or more J gene segments; B is optional and encodes one or more characterised binding domains of known specificity; C encodes a CH2-CH3 dimerisation domain; and D encodes one or more characterised binding domains of known specificity.

The characterised binding domains encoded by B and D may be of the same or different specificity.

A "VH binding complex locus" in the context of the present invention relates to a minimal locus defined by the formula 5'-A-B-C-D-3' wherein: A comprises one or more V gene segments, one or more D gene segments and one or more J gene segments; B is optional and encodes one or more characterised binding domains of known specificity; C encodes a CH2-CH3 dimerisation domain; and D encodes one or more characterised binding domains of known specificity. Preferably, the primary source of VH binding complex repertoire variability is the CDR3 region formed by the selection of V, D and J gene segments and by the V-D and D-J junctions with additional diversity imparted by mutations acquired as a result of affinity maturation.

The VH binding complex will be at least tetravalent and bispecific. In each locus, A will provide a VH binding domain at the N terminal end of each chain of each homodimeric VH binding complex. Thus, the N terminal end of each VH binding complex will be bivalent, as it will provide two binding sites specific for the antigen with which the mammal was challenged. In each locus, D will provide a characterised binding domain at the C terminal end of each chain of each homodimeric binding complex and thus the C terminal end of the homodimeric VH binding complex will be bivalent.

As explained below, each VH binding complex locus may include further coding sequences between A and D. These extra sequences may encode further characterised binding domains and so each homodimeric VH binding complex may be multivalent and bispecific or multivalent and multispecific, depending on the number and specificities of the characterised binding domains.

Preferably, in each locus, A comprises one or multiple V gene segments, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32,

33, 34, 35, 36, 37, 38, 39, 40, 45, 50 or 60 V gene segments, which may be derived from any vertebrate species.

In one embodiment, in each locus A comprises only one V gene segment.

5 Alternatively, in each locus, A comprises more than one V gene segment. In one alternative of this embodiment, each V gene segment is different from all other V gene segments. In a second alternative, each V gene segment is identical to all the other V gene segments. In this second alternative, the remaining gene segments in A in each locus may be the same as or may be different from those in all the other loci.

10 It is envisaged that the non-human mammal may contain multiple copies of a single VH binding complex locus. This has the advantage of optimising the chances that a productive re-arrangement in a B cell will take place, thus allowing the production of a useful VH binding complex.

If the non-human mammal contains a number of different VH binding complex loci, this will further optimise the chances of obtaining a VH binding complex with a desired specificity.

15 As noted above, each A may comprise multiple V gene segments. In this embodiment, the V gene segments in any one A may all be derived from an organism of the same species, e.g. all V gene segments may be of human origin. Alternatively, the V gene segments in any one A may be derived from organisms of different species, e.g. some V gene segments from human and others from a camelid or from a shark. Preferably, the V gene segments are of human
20 origin.

The term 'V gene segment' encompasses any naturally occurring V gene segment derived from a vertebrate, including camelids and human. The V gene segment must be capable of recombining with a D gene segment, a J gene segment and a gene segment encoding the CH2-CH3 dimerisation domain (which may optionally include a hinge exon and/or aCH4 exon, but
25 excludes a CH1 exon) to generate a VH binding complex when the nucleic acid is expressed.

A V gene segment includes within its scope any naturally occurring or engineered gene sequence encoding a natural V sequence, a homologue, a derivative or a protein fragment which is capable of recombining with a D gene segment, a J gene segment and a gene segment encoding the CH2-CH3 dimerisation domain to generate a VH binding complex as

defined herein. A V gene segment may, for example, be derived from a T cell receptor locus or an immunoglobulin light chain locus.

Preferably, if there are multiple VH binding complex loci, these may comprise any number or combination of the 39 functional human V gene segments and engineered variants thereof
5 with improved solubility properties distributed across the multiple loci. These may be on any number of loci, e.g. four loci comprising eight V gene segments plus one locus comprising seven V gene segments; seven loci comprising four V gene segments plus one locus comprising three V gene segments; or thirty-nine loci comprising one V gene segment each.

A V gene segment must be capable of recombining with a D gene segment and a J gene
10 segment to form a VH binding domain. The VH binding domain will be linked to the CH2-CH3 dimerisation domain, which in turn is linked to a characterised binding domain.

Human heavy chain V gene segments are classified into seven families, V1 to V7, and the individual genes within each family numbered. The frequency at which each gene is used is dependent on the varying requirements of the particular immune response. For example, the
15 genes of family V3 may be preferentially used in comparison to those of family V5 when responding to bacterial antigens. Therefore, in a further preferred embodiment of the invention, groups of V gene segments which have been shown to be useful for generating an antibody response against specific antigens are grouped into separate lines of transgenic non-human mammals. The V gene segments may be grouped according to family or they may be
20 grouped according to individual function. For example, if the V gene segments of family V3 are shown to be useful for generating an immune response against bacterial antigens, then these may be used to generate a transgenic non-human mammal which is particularly useful for generating diverse VH binding complexes against bacterial antigens. Alternatively, if it is shown that several individual genes from families V3 and V5 are useful for generating an
25 immune response against bacterial antigens, then these may be grouped together and used to generate a transgenic non-human mammal which is particularly useful for generating diverse VH binding complexes against bacterial antigens.

In the context of the present invention, the terms 'a D gene segment' and 'a J gene segment' include naturally occurring and engineered sequences of D and J gene segments. Preferably,
30 the D and J gene segments are derived from the same vertebrate from which the V gene segment(s) is (are) derived. For example, if a V gene segment is derived from a human, then

the D and J gene segments, whether solubilised or engineered, are preferably also derived from a human. Alternatively, if the V gene segments are derived, for example, from a camelid and optionally humanised, then the D and J gene segments may be derived from a human or camelid, dependent on the desired product.

- 5 The terms D gene segment and J gene segment also include within their scope derivatives, homologues and fragments thereof as long as the resultant segment can recombine with the remaining components of the VH binding complex locus as herein described to generate a homodimeric VH binding complex as herein described. D and J gene segments may be derived from naturally occurring sources or they may be synthesised using methods familiar
- 10 to those skilled in the art and described herein. The V, D and J gene segments are capable of recombination and preferably undergo somatic mutation.

The D and J gene segments are preferably derived from a single vertebrate species. This may be any vertebrate species but is preferably a human.

- Preferably, A of each VH binding complex locus comprises from one to forty (2, 3, 4, 5, 6, 7,
- 15 8, 9, 10, 12, 14, 16, 18, 20, 30 or 40) or more D gene segments. The D gene segments may be derived from any vertebrate species but, most preferably, the D gene segments are human D gene segments (normally 25 functional D gene segments).

- Preferably, A of each VH binding complex locus comprises from one to twenty (2, 3, 4, 5, 6,
- 20 7, 8, 9, 10, 12, 14, 16, 18 or 20) or more J gene segments. The J gene segments may be derived from any vertebrate species but, most preferably, the J gene segments are human J gene segments (normally 6 J gene segments).

- A of each VH binding complex locus may contain the same D and J gene segments. Alternatively, A of each VH binding complex locus may contain different combinations of D and J gene segments. For example, where each VH binding domain locus contains only one
- 25 V gene segment and this segment is identical in each locus, it is then advantageous to use different combinations of D and J gene segments in each locus to further optimise the chances of obtaining a productive re-arrangement. However, where A of each VH binding complex locus contains one or more different V gene segments, it may be advantageous to use the same combination of D and J gene segments in each locus.

Preferably, A of each VH binding complex locus comprises one or more V gene segments, twenty five functional human D gene segments and six human J gene segments.

In the context of the present invention, the binding domain encoded by D may comprise binding function alone, or may incorporate additional functionality such as a selective marker enzyme or fluorescent marker, or maybe tagged to facilitate subsequent product purification.

According to a further preferred embodiment of the first aspect of the invention, the region of the VH binding complex locus defined by D encodes more than one characterised binding domain of known specificity.

Each carboxyl terminus characterised binding domain encoded by D has known binding specificity and may comprise any polypeptide chain capable of recognising and binding an epitope on a target antigen. Preferably the binding domain is a VH domain. Alternatively, the binding domain may be an engineered scFv binding domain, a receptor binding protein, a fibronectin binding domain, a coiled coil region, an arginine finger, a leucine zipper domain, a cadherin repeat, a death effector domain (DED), an immunoglobulin-like domain, a phosphotyrosine binding domain (PTB), a Pleckstrin homology domain (PH), an SH2 or SH3 domain, a zinc finger domain (see also http://pawsonlab.mshri.on.ca/index.php?option=com_content&task=view&id=30&Itemid=63 /). Where there is more than one binding domain, the binding domains may bind the same or different antigens, or different epitopes on the same antigen.

According to a further preferred embodiment of the first aspect of the invention, B of the VH binding complex locus, which encodes one or more characterised binding domains of known specificity, is present. Each binding domain encoded by B has known binding specificity and may comprise any polypeptide chain capable of recognising and binding an epitope on a target antigen. Preferably the binding domains are VH domains. Alternatively, the binding domain may be a scFv binding domain, a receptor binding protein, a fibronectin binding domain, a coiled coil region, an arginine finger, a leucine zipper domain, a cadherin repeat, a death effector domain (DED), an immunoglobulin-like domain, a phosphotyrosine binding domain (PTB), a Pleckstrin homology domain (PH), an SH2 or SH3 domain, a zinc finger domain (see above). Where there is more than one binding domain, the binding domains may bind the same or different antigens or different epitopes on the same antigen. Binding domains may have the same or different specificities and affinities for antigens.

Optionally the gene segments B and D of the said VH binding complex gene locus have been engineered to eliminate nucleotide sequence motifs recognised by the AIDS enzyme (AGCT). This strategy minimises the likelihood of inappropriate affinity maturation concomitant with the desired affinity maturation of the A of the VH binding complex following VDJ rearrangement and antigen stimulated B-cell activation.

In the context of the present invention, the term 'heterologous' means a nucleotide sequence or a locus as herein described which is not endogenous to the mammal in which it is located.

Preferably, each locus contains the same CH2-CH3 dimerisation domain. Optionally, different loci comprise the same CH2-CH3 dimerisation domain, each comprising differing characterised amino- and carboxyl-terminal binding domains.

Optionally, C further encodes a hinge domain and/or a CH4 domain. C does not encode a CH1 domain. Preferably, C encodes only the CH2-CH3 domain.

The CH2-CH3 dimerisation domain, as encoded by C, may be encoded by a naturally occurring or engineered gene segment that is capable of recombining with a V gene segment, a D gene segment and a J gene segment in a B cell resulting in the synthesis of a homodimeric tetravalent VH binding complex. When additional binding domains are present at either amino and/or carboxyl termini of the dimerisation domain, a multivalent VH binding complex is produced.

The CH2-CH3 dimerisation domain may be any immunoglobulin heavy chain constant region dimerisation domain (devoid of CH1), resulting in the synthesis and secretion by B cells of a homodimeric tetravalent VH binding complex. If additional amino- and/or carboxy-terminal characterised binding domains are present, the homodimeric VH binding complex may be of greater complexity, dependent on the configuration of the VH binding complex locus or loci expressed in the non-human transgenic mammal.

Engineering of individual CH2-CH3 dimerisation domains, optionally including a hinge domain and/or a CH4 domain (as in IgM), can also result in the addition or deletion of functionality (Van Dijk and van der Winkel, Curr. Opin. Chem. Biol., (2001) Aug 5 (4), 368-374). Thus, the inclusion of IgA constant region functionality might provide improved mucosal function against pathogens, whilst the presence of IgG1 constant region functionality provides enhanced serum stability in vivo.

The presence of heavy chain CH2 and CH3 constant domains provides the basis for stable dimerisation as seen in natural antibodies and provides recognition sites for post-translational glycosylation. The presence of CH2 and CH3 also allows for secondary antibody recognition when the homodimeric VH binding complexes are used as reagents and diagnostics.

- 5 Preferably, the CH2-CH3 dimerisation domain is of human origin, in particular when the VH binding complexes are to be used for therapeutic applications in humans. Where the VH binding complexes are to be used for veterinary purposes, the CH2-CH3 dimerisation domain is preferably derived from the target organism, vertebrate or mammal, in or on which veterinary therapy is to be performed (e.g. cow, pig, horse, chicken, sheep, goat, etc.).
- 10 A "CH2-CH3 dimerisation domain" as herein defined is devoid of CH1 but preferably includes the sequences present in naturally occurring CH2-CH3 domains of vertebrates, especially mammals. This varies in a class specific manner. For example, IgG and IgA are naturally devoid of a CH4 domain, but IgM and IgE will also comprise a CH4 domain. The term "CH2-CH3 dimerisation domain" also includes within its scope derivatives, homologues
- 15 and fragments thereof in so far as the "CH2-CH3 dimerisation domain" is able to form a functional dimerisation domain, with associated desired effector functions, and is capable of combining with a VH domain as a result of VDJ rearrangement to form a homodimeric VH binding complex.

The CH2-CH3 dimerisation domain may be engineered to be free of functional domains, for

20 example the carboxy-terminal CH4 domain, provided that engineering does not affect secretory mechanisms, prevent cell surface assembly or B cell activation and maturation. Additional features maybe engineered into the locus, for example to improve glycosylation or add function.

- Preferably, each VH binding complex locus includes one or more regions encoding a hinge
- 25 domain or a flexible hinge-like peptidic linker. Such one or more regions will be located in each locus such that the homodimeric VH binding complex, when expressed, has a hinge domain or a flexible hinge-like peptidic linker linking any one, selected ones or all of the pairs of adjacent domains referred to above. In particular, preferably in the homodimeric VH binding complex as expressed, there will be a hinge domain or a hinge-like peptidic linker
- 30 linking the N terminal VH binding domain to the CH2-CH3 dimerisation domain and/or linking the CH2-CH3 dimerisation domain to the C terminal characterised binding domain.

Preferably, each VH binding complex locus further comprises C' located between A and B, where C' encodes at least one CH2-CH3 dimerisation domain as defined above (i.e. such that each locus is defined by the formula 5'-A-C'-B-C-D-3'). The homodimeric VH binding complex produced by antigen challenge of a non-human mammal containing such a preferred locus will be produced as a result of CH2-CH3 dimerisation domain class switching. The preferred CH2-CH3 dimerisation domain encoded by part C' is C μ . In this embodiment of the invention, following antigen challenge, a homodimeric VH binding complex based on IgM is initially expressed as a membrane bound form as a result of VDJ rearrangement, allelic exclusion and B cell activation. Class switching and differential splicing then results in the production of tetravalent or multivalent homodimeric VH binding complexes (dependent on the complexity of the VH binding complex loci) which are then synthesised and secreted from B cells in the given non-human transgenic mammal.

Preferably, each locus comprises a gene segment encoding a recombinase recognition site (such as lox) located between A and C' and between C' and B (if present) or between C' and C (if B is not present). Thus, activation of a recombinase in plasma cells in vivo or subsequently in hybridoma cells in vitro would result in the elimination of C' and expression of a downstream CH2-CH3 dimerisation domain in position C in the locus (see Figure 4).

The transgenic non-human mammal is preferably a rodent such as a rabbit, guinea pig, rat or mouse. Mice are especially preferred. Alternative mammals such as pigs, goats, sheep, cats, dogs or other animals may also be employed. Preferably transgenic non-human animals are generated using established oocyte injection technology alone. Where established, ES cell technology or cloning may also be used.

Advantageously, immunoglobulin heavy and optionally light chain loci endogenous to the mammal are deleted or silenced when a heterologous VH binding domain locus or loci are expressed according to the methods of the invention. Alternatively one or both of the endogenous immunoglobulin gene loci maybe engineered to encode a VH binding complex of the invention using homologous recombination as opposed to transgenesis as a route to introduce a functional VH binding complex. Endogenous constant regions may be used with the proviso that the CH1 domain has been deleted preferably during the construction of the VH binding complex loci. Alternatively, but less preferably, in non-human mammals lacking light chain functionality, the CH1 domain may be left intact. Undefined natural mechanisms will then result in the derivation of VH binding complex mRNA transcripts which lack CH1

functionality (see Zou X., Osborn M.J., Bolland D.J., Smith J.A., Corcos D., Hamon M., Oxley D., Hutchings A., Morgan G., Santos F., Kilshaw P.J., Taussig M.J., Corcoran A.E., Brüggemann M., Heavy chain-only antibodies are spontaneously produced in light chain-deficient mice, *J. Exp. Med.*, 2007;204:3271-83). Accordingly, a further aspect of the invention provides a transgenic non-human mammal comprising more than one heterologous homodimeric VH binding complex locus as defined above. It has been previously shown (GB0618345.3 and Janssens [supra]) that allelic exclusion determines which of multiple VH binding complex loci present in a non-human transgenic animal is productively expressed in the given activated B cell. Thus, the invention incorporates the presence of multiple VH binding complex loci. These may be the same, so increasing the likelihood of a productive event. Alternatively they may be different. For example they may have different VH domains or different constant regions or different (in quantity and quality) binding domains. Furthermore, B-C-D might be varied so that different loci comprise a common dimerisation domain C but differ in the number and specificity of the binding domains (encoded by B and D). Thus, a single non-human transgenic mammal comprising multiple and different loci may be programmed to provide diverse homodimeric VH binding complexes in response to challenge with single or multiple antigens.

Homodimeric VH binding complex-producing cells may be derived from transgenic non-human mammals as defined herein and used, for example, in the preparation of hybridomas for the production of homodimeric VH binding complexes as herein defined. In addition or alternatively, nucleic acid sequences may be isolated from these transgenic non-human mammals and used to produce monovalent VH binding domains or homodimeric VH binding complexes using library display, expression systems and recombinant DNA techniques which are familiar to those skilled in the art.

Alternatively or in addition, antigen-specific VH binding domains and homodimeric VH binding complexes may be generated by immunisation of a transgenic non-human mammal as defined herein.

Accordingly, the invention also provides a method for the production of VH binding domains by immunising a transgenic non-human mammal as defined above with an antigen. Monospecific VH binding domains and homodimeric VH binding complexes may be isolated, characterised and manufactured using well-established methods known to those skilled in the art.

According to a second aspect of the invention there is provided a source of affinity matured antigen-specific VH binding domains derived as amino terminal VH binding domains from the VH binding complexes produced in response to antigen challenge in the first aspect of the invention. Such VH binding domains are preferably human and maybe used as a basic building block for the derivation of diverse VH binding molecules encompassing VH binding domains alone or more complex binding complexes as envisaged by Ward et al., ((1989) Nature, 341, 544-546).

A "VH domain" in the context of the present invention refers to an expression product of a V gene segment when recombined with a D gene segment and a J gene segment as defined above. Preferably, the VH domain has improved solubility and ability to bind antigen as a result of VDJ recombination and somatic mutation. There is no dependency on the presence or absence of the enlarged CDR3 loop peculiar to the camelid species. The VH domain is able to bind antigen as a monomer.

The VH domain coding sequences may be derived from a naturally occurring source or they may be synthesised using methods familiar to those skilled in the art.

The properties of the VH domain may be altered or improved by selecting or engineering V, D and/or J gene segments which encode sequences with the required characteristics. Some of the 39 functional human V segments may not be suitable for the production of VH binding domains. Studies have been carried out in the prior art in an attempt to improve VH binding domain characteristics (see Jespers L., Schon O., Famm K., Winter G., (2004) Aggregation-resistant domain antibodies selected on phage by heat denaturation, Nat. Biotechnol., 22(9):1161-5). With regard to specific VH region characteristics, others have used phage display techniques to generate VH binding domains showing improved stability in the harsh conditions associated with anti-dandruff shampoo (Dolk et al., (2005) Appl. Environ. Microbiol., 71(1):442-50). Preferably natural improvements occur in vivo as a result of affinity maturation in vivo during B cell maturation, allowing selection of high affinity VH binding domains and complexes from hybridomas secreting favourable yields of soluble product. V segments with preferred characteristics resulting from affinity maturation may be introduced into new VH binding domain loci for the subsequent enhanced production of antigen-specific VH binding domain complexes.

The methods of generating VH binding domains as described in the above aspects of the invention may be of particular use in the generation of human VH binding domains and VH binding complexes for human therapeutic use, as often the administration of antibodies to a species of vertebrate which is of different origin from the source of the antibodies results in the onset of an immune response against those administered antibodies. The VH binding domains produced according to the invention have the advantage over those of the prior art in that they are of substantially a single class of VH binding domain or homodimeric VH binding domain complex preferably representing a product without the need for further downstream engineering or optimisation. Products are preferably of human origin, but may be designed to incorporate features from any species dependent on their final use. VH binding domains are of high affinity resulting from a combination of VDJ recombination and affinity maturation in vivo.

VH binding domains alone and homodimeric VH binding complexes of the invention, especially those of human origin, have wide ranging applications in the field of healthcare as medicines, imaging agents and diagnostics, and more widely for research, industrial, agricultural and environmental purposes and in the food industry.

VH binding domain

An antigen-specific VH binding domain of the invention may be cloned from, e.g., said VH binding complex mRNA isolated from an antibody-producing cell of an immunised transgenic mammal as described above. Cloned VH binding domain sequences can also be isolated from mRNA arrays (Ward et al., (1989) Nature, 341, 544-546) or similar array libraries, for example using yeast-based systems (Boder and Wittrup, (1997) Nat. Biotechnol., 15, 553-7). Antigen-specific VH binding domains or homodimeric VH binding complexes can then be manufactured in scalable bacterial, yeast or alternative expression systems.

Where the homodimeric VH binding complex is isolated from a characterised hybridoma as a result of antigen challenge, the amino terminal VH binding domain sequence derived from mRNA can be directly cloned into an expression vector without recourse to additional selection steps necessary using phage and other display systems to characterise and optimise the affinity of the selected VH binding domain.

Production systems for homodimeric VH binding complexes include yeast, mammalian cells in culture (e.g. B cell hybridomas, CHO cells), plants (e.g. maize), transgenic goats, rabbits,

pigs, cattle, sheep and chickens, also insect larvae suited to mass rearing technology. Other production systems, including virus infection (e.g. baculovirus in insect larvae and cell lines), are alternatives to cell culture and germline approaches. Other production methods will also be familiar to those skilled in the art. Suitable methods for the production of VH binding domains alone are known in the art. For example camelid VHH binding domains have been produced in bacterial systems (Olichon A., Schweizer D., Muyldermans S., de Marco A., Heating as a rapid purification method for recovering correctly-folded thermotolerant VH and VHH domains. BMC Biotechnol., 2007;7:7 and references therein) and VH binding domains have been produced in yeast and mammalian culture systems (see WO01/90192).

- 10 Insect larvae from transgenic fly lines have been shown to produce VH binding domains with characteristics indistinguishable from the same antibody produced by mammalian cells (WO2004/013171).

The present invention also provides a vector(s) including DNA sequences encoding VH binding complex loci as defined above, VH binding domains as defined above or VH binding complexes as defined above.

The present invention also provides a host cell transformed with vectors according to the present invention.

- 20 The presence of two or more identical VH domains acting in a co-operative manner provides a VH binding complex of greater affinity and avidity than a single VH alone. A tetravalent bispecific VH binding complex can facilitate cross-linking of different targets whilst retaining the beneficial cooperative effect of two VH binding domains for each antigen. For example, a tetravalent bispecific VH binding complex may be utilised to enhance cell-cell interactions or cell-pathogen interactions. In this embodiment, the VH binding complexes of the invention can be utilised, for example, to bridge between two cell types such as an erythrocyte and a pathogen (see Taylor et al., (1991) PNAS 88, 3305-3309). Bispecificity can be used to simultaneously inhibit two components of an enzyme pathway (Jendreyko et al., (2003) J. Biol. Chem., 278, 47812-47819).

- 30 Bispecificity can also be used to bring an effector moiety into close proximity with a target cell. The VH binding domains at the amino terminal end of each complex are identical and preferably those at the carboxyl terminal end are identical (but engineered in the VH binding complex locus to recognise a different antigen or epitope to that at the amino terminal end),

facilitating co-operative binding of pairs of VH binding domains. The possibility of more than one binding domain at the amino or carboxyl terminus will further increase binding affinity where binding domains are identical. Where binding domains are different, this has advantages for anti-infective strategies through reducing the likelihood of viral or bacterial escape through mutation changing the conformation of more than one epitope.

The term 'effector moiety' as used herein includes any moiety that mediates a desired biological effect on a cell. The effector moiety is preferably soluble and may be a peptide, polypeptide or protein or may be a non-peptidic structure. For example, the effector moiety may be an enzyme, hormone, cytokine, drug, pro-drug, toxin, in particular a protein toxin, a radionuclide in a chelating structure, an imaging agent, albumin or an inhibitory agent. The effector moiety may be a cell, for example a T cell, a peptide, polypeptide or protein or may be a non-peptidic structure. The effector moiety associated with the VH binding domain may be cellular, proteinaceous, organic or inorganic in nature, dependent on the desired effect.

Albumin, immunoglobulins or other serum or cell surface proteins may be utilised as an effector moiety to increase the stability or pharmacokinetic and/or pharmacodynamic properties of the antigen specific VH binding domain (Sung et al., (2003) J. Interferon Cytokine Res., 23 (1): 25-36; Harmsen et al., (2005) Vaccine, 23 (41) 4926-4934). Alternatively, the effector moiety may be a PEGylated structure or a naturally glycosylated structure so as to improve pharmacodynamic properties.

Polypeptide Dimerisation Domains

The present inventors have also recognised that the properties of any VH binding complex are not just dependent on the VH binding domain and other binding domains incorporated in the final binding complex. The CH2-CH3(-CH4) dimerisation domain, may comprise additional effector activity(s), dependent on which class of immunoglobulin heavy chain (or subclass) it is derived from, e.g. IgM, IgA or IgG. Dimerisation domains are linked covalently to binding domains at the dimerisation domains' amino and carboxyl termini, preferably via a hinge domain or flexible hinge-like peptidic linker.

Optionally, the VH binding domain complex includes natural hinge domains or engineered flexible hinge-like peptidic linkers linking the VH binding domains, alternative binding domains and the dimerisation domain. The presence of hinge domains facilitates the

independent function of the VH binding domains and alternative binding domains in the resultant VH binding complex.

5 Optionally the CH2-CH3 dimerisation domain may comprise other useful functions, or may be engineered to incorporate additional features such as recognition sequences for glycosylation, pegylation, cell surface receptor binding, or tags for antibody or binding protein recognition. Dimerisation domains may be engineered to optimise association through the introduction or elimination for example of additional cysteine residues.

10 The term 'binding domain' as used herein in respect of all the above aspects of the present invention includes any binding domains (including VH binding domains) that have effector activity in a physiological medium. Such a binding domain must also have the ability to bind to a target under physiological conditions.

15 A VH binding domain may comprise a camelid VHH domain or may comprise a VH domain obtained from a non-camelid. Alternatively the VH binding domain may comprise, for example, a camelid V segment and human D and J segments. Preferably, the VH binding domain comprises entirely human sequences.

20 Alternative binding domains include domains that can mediate binding or adhesion to a cell surface. Suitable domains which may be used in the polypeptide complexes of the invention are mammalian, prokaryotic and viral cell adhesion molecules, cytokines, growth factors, receptor antagonists or agonists, ligands, cell surface receptors, regulatory factors, structural proteins and peptides, serum proteins, secreted proteins, plasmalemma-associated proteins, viral antigens, bacterial antigens, protozoal antigens, parasitic antigens, lipoproteins, glycoproteins, hormones, neurotransmitters, clotting factors and the like, and engineered single chain Fvs.

Polynucleotide sequences, vectors and host cells

25 The present invention also provides a polynucleotide sequence encoding any one of the VH binding complexes of the present invention, a vector comprising one or more of the polynucleotide sequences referred to above and a host cell transformed with a vector or vectors encoding the polypeptide binding complex of the present invention. The polynucleotides preferably include sequences which allow the expressed VH binding complex
30 to be secreted into the medium in which the host cell is growing. The host cell may include

but is not limited to bacterial and yeast, insect, plant and mammalian host cells. The VH binding complex may also be retained in the host cell with intracellular or “intrabody” function.

Furthermore, the present invention provides a transgenic non-human mammal expressing at least one homodimeric VH binding complex derived as a result of antigen challenge of the present invention.

The production of homodimeric VH binding complexes for healthcare applications requires large scale manufacturing systems, examples of which are discussed in detail above. Such systems include plants (e.g. maize), transgenic cattle and sheep, chickens and insect larvae suitable for mass rearing technology. Other production systems, including virus infection (e.g. baculovirus in insect larvae and cell lines) as an alternative to cell culture and germ line approaches will also be familiar to those skilled in the art.

These methods and other suitable methods known in the art can be used.

Uses of Soluble VH binding domains and homodimeric VH binding complexes of the Invention

Soluble VH binding domains and homodimeric VH binding complexes of the invention have a great number of applications and are particularly advantageous, e.g. as therapeutics for the treatment, prevention and diagnosis of diseases. They are also useful for cytochemical labelling, targeting methods, therapy and diagnostics.

In mono-antibody therapy, pathogen escape, for example due to a mutation leading to loss of a single binding site, will abolish the therapeutic effect of the antibody. The production of homodimeric VH binding complexes recognising different antigens on the same pathogen can overcome this problem. The use of two or more VH binding domains having different specificities in the homodimeric VH binding complexes of the invention can also be utilised to enhance both cell-cell interactions and cell-pathogen interactions. The introduction of further binding domains at amino and carboxy termini of homodimeric VH binding complexes further enhances the therapeutic value of such homodimeric VH binding complexes.

In one embodiment, the soluble homodimeric VH binding complexes of the invention can be utilised, for example, to bridge between two cell types such as a pathogen and an erythrocyte or a macrophage, or a tumour cell and a T cell. Alternatively, the complex may recognise two or more epitopes on the same pathogen with effector function being provided by a receptor recognition domain within the CH2-CH3 dimerisation domain or inserted between the dimerisation domain and the hinge sequence. Soluble homodimeric VH binding complexes can also be used to target two components of a single disease related pathway simultaneously, for example, two different cytokines or cytokine receptors which modulate inflammatory responses such as TNFa and Il-6.

Alternatively, the homodimeric VH binding domain complexes may be used to target cells and tissues in vivo, then subsequently to capture circulating effector molecules or imaging agents. For example, bispecific tumour targeting agents can be used to capture pro-drug converting complexes for the subsequent localised conversion of pro-drug to reactive agent. Bi- and multi- specific VH binding complexes in combination with effector agents may also be used to bind and destroy one or more pathogens dependent on the selection of binding domains. Alternatively, the presence of two or more binding domains which recognise different antigens on the same pathogen provide clinical advantages and reduce the likelihood of pathogen escape and drug redundancy as a result of mutation within the pathogen.

Soluble VH binding domains and homodimeric VH binding complexes comprising predominantly human sequences are suitable for pharmaceutical use in humans, and so the invention provides a pharmaceutical composition of a homodimeric VH binding complex, preferably comprising VH binding domains linked to a dimerisation domain through hinge domains at the amino and carboxyl termini. The invention also provides the use of a homodimeric VH binding complex of the present invention in the preparation of a medicament for the prophylaxis and/or treatment of disease. Where appropriate, VH binding complexes and effector moieties may be formulated separately or together.

The pharmaceutical compositions and medicaments will typically be formulated before administration to patients.

For example, the homodimeric VH binding complexes may be mixed with stabilisers, particularly if they are to be lyophilised. Addition of sugars (e.g. mannitol, sucrose or trehalose) is typical to give stability during lyophilisation, and a preferred stabiliser is

mannitol. Human serum albumin (preferably recombinant) can also be added as a stabiliser. Mixtures of sugars can also be used, e.g. sucrose and mannitol, trehalose and mannitol, etc.

Buffer may be added to the composition, e.g. a Tris buffer, a histidine buffer, a glycine buffer or, preferably, a phosphate buffer (e.g. containing sodium dihydrogen phosphate and disodium hydrogen phosphate). Addition of buffer to give a pH between 7.2 and 7.8 is preferred, and in particular a pH of about 7.5 is preferred.

For reconstitution after lyophilisation, sterile water for injection may be used. It is also possible to reconstitute a lyophilised cake with an aqueous composition comprising human serum albumin (preferably recombinant).

10 Generally, the homodimeric soluble VH binding complexes will be utilised in purified form together with pharmacologically appropriate carriers.

The invention thus provides a method for treating a patient, comprising administering a pharmaceutical composition of the invention to the patient. The patient is preferably a human, and may be a child (e.g. a toddler or infant), a teenager or an adult, but will generally be an adult.

The invention also provides soluble VH binding domains and homodimeric VH binding complexes of the invention for use as a medicament.

The invention also provides the use of the soluble VH binding domains and homodimeric VH binding complexes of the invention in the manufacture of a medicament for treating a patient.

20 These uses, methods and medicaments are preferably for the treatment of one of the following diseases or disorders: wound healing, cell proliferative disorders, including neoplasm, melanoma, lung, colorectal, osteosarcoma, rectal, ovarian, sarcoma, cervical, oesophageal, breast, pancreas, bladder, head and neck and other solid tumours; myeloproliferative disorders, such as leukemia, non-Hodgkin lymphoma, leukopenia, thrombocytopenia, angiogenesis disorder, Kaposi's sarcoma; autoimmune/inflammatory disorders, including allergy, inflammatory bowel disease, arthritis, psoriasis and respiratory tract inflammation, asthma, immunodisorders and organ transplant rejection; cardiovascular and vascular disorders, including hypertension, oedema, angina, atherosclerosis, thrombosis, sepsis, shock, reperfusion injury and ischemia; neurological disorders including central nervous system

disease, Alzheimer's disease, brain injury, amyotrophic lateral sclerosis, and pain; developmental disorders; metabolic disorders including diabetes mellitus, osteoporosis, and obesity, AIDS and renal disease; infections including viral infection, bacterial infection, fungal infection and parasitic infection, pathological conditions associated with the placenta and other pathological conditions and for use in immunotherapy.

In a further aspect still, the present invention provides the use of soluble VH binding domains and homodimeric VH binding complexes of the present invention as a diagnostic, prognostic or therapeutic imaging agent.

The present invention provides the use of soluble VH binding domains and homodimeric VH binding complexes as herein described as an abzyme.

The present invention also provides the use of soluble VH binding domains and homodimeric VH binding complexes according to the present invention as enzyme inhibitors or receptor blockers.

The present invention also provides the use of soluble VH binding domains and homodimeric VH binding complexes for use as a therapeutic, imaging agent, diagnostic, abzyme or reagent.

The present invention also provides soluble VH binding domains and homodimeric VH binding complexes for use as an intracellular binding agent (intrabody), and provides vectors functional in target cells for the intracellular expression of intrabodies comprising VH binding domains and homodimeric VH binding domain complexes.

The invention is now described, by way of example only, in the following detailed description which refers to the following figures. The skilled person will appreciate that modification of details may be made without departing from the scope of the invention. All documents cited herein are incorporated by reference in their entirety.

FIGURES

Figure 1 shows the strategy for the design and construction of a VH binding domain locus which results in the expression, in mature B cells, of a transgenic non-human mammal of an affinity matured tetravalent bispecific VH binding domain. The dimerisation domain comprises a heavy chain CH2-CH3 constant region devoid of CH1. The amino terminal VH binding domain is derived by VDJ rearrangement and affinity maturation as a result of

antigen challenge and linked via a hinge region to the dimerisation domain. The carboxyl terminal binding domain (BD) is of pre-determined binding specificity linked via a hinge or flexible hinge-like domain to the dimerisation domain and encoded within the VH binding domain locus. on the locus incorporates the natural gene segments M1 and M2 required for the process of switching in an immunoglobulin locus from the membrane bound form of the antibody to the secreted form of the antibody.

Figure 2 shows the strategy to recombine the additional hinge and binding domain into an immunoglobulin locus such as generated in PCT/GB2005/002892 and PCT/IB2007/00347. Preferably the strategy is based on cloning the hinge and a VH domain of known binding specificity into a recombination vector by standard methods and PCR amplification followed by standard cloning.

Figure 3 shows the strategy for the design and construction of a VH binding domain locus which results in the expression, in mature B cells, of a transgenic non-human mammal of an affinity matured tetravalent bispecific VH binding domain and a marker protein. The dimerisation domain comprises a heavy chain CH2-CH3 constant region devoid of CH1. The amino terminal VH binding domain is derived by VDJ rearrangement and affinity maturation as a result of antigen challenge and linked via a hinge region to the dimerisation domain. The marker protein (e.g. GFP) is recombined onto the CH3 domain followed by a carboxyl terminal binding domain (BD) of predetermined binding specificity linked via a hinge or flexible hinge-like domain to the marker protein domain and encoded within the VH binding domain locus. The locus incorporates the natural gene segments M1 and M2 required for the process of switching in an immunoglobulin locus from the membrane bound form of the antibody to the secreted form of the antibody.

Figure 4 shows the strategy for the design and construction of a VH binding domain locus which results in the expression, in mature B cells, of a transgenic non-human mammal of an affinity matured tetravalent bispecific VH binding domain and an effector protein. The dimerisation domain comprises a heavy chain CH2-CH3 constant region devoid of CH1. The amino terminal VH binding domain is derived by VDJ rearrangement and affinity maturation as a result of antigen challenge and linked via a hinge region to the dimerisation domain. The effector protein (e.g. NTR) is recombined onto the CH3 domain followed by a carboxyl terminal binding domain (BD) of pre-determined binding specificity linked via a hinge or flexible hinge-like domain to the marker protein domain and encoded within the VH binding

domain locus. The locus incorporates the natural gene segments M1 and M2 required for the process of switching in an immunoglobulin locus from the membrane bound form of the antibody to the secreted form of the antibody.

Figure 5 shows the strategy for the inclusion of additional amino, or carboxyl binding domains with pre-determined characteristics for the derivation of VH binding complexes. Only the amino terminal VH binding domain is derived from VDJ rearrangement and affinity maturation as a result of antigen challenge.

EXAMPLE 1

- 10 Generation of a germline locus to generate tetravalent bi-specific VH homodimers containing at the C-terminus an additional binding domain (BD). In this example the C terminal binding domain (BD) is a specific human VH.

The general scheme to generate such loci and how such a locus leads to the production of a tetravalent bi-specific VH homodimer is shown in Figure 1. The scheme is essentially a
15 modification of that used to generate heavy only antibodies in response to antigen challenge using both natural and /or engineered V segments (see PCT/GB2005/002892 and PCT/IB2007/00347 incorporated by reference and case histories). The additional C-terminal VH domain (BD in Figure 1) is recombined into an heavy chain only antibody locus (e.g. IgG2) locus contained present in a PAC (see e.g. patent P044839WO, Figure 1 line 1) at a
20 position just downstream of the splice donor site of the CH3 exon, but before the stop codon TGA (Figure 1 lines 2 and 3) resulting in the locus shown in Figure 1 line 4. The newly generated locus is introduced into transgenic mice. The resulting modified mice are immunized with a specific antigen. This will result in the membrane bound IgG specifically against the antigen by the normal route of VDJ recombination and the translation of an
25 mRNA transcript that was generated by the normal splicing from the exons coding for VDJ to hinge to CH2 to CH3 to M1 and M2 (Figure 1 line 2). Upon the normal switching after antibody maturation (by hypermutation) from a membrane bound to a secreted form of the antibody by a lack of splicing from CH3 to M1 but use of the poly A site in the intron between CH3 and M1. Instead of terminating the protein coding at the normal TGA stop
30 codon the novel hinge and extra VH domain will be incorporated into the mature mRNA..In the first step the hinge and a VH domain of known binding specificity are cloned into a

recombination vector by standard methods using the oligonucleotides that are homologous to the 3' end of the CH3 exon shown in Figure 2 and PCR amplification followed by standard cloning. This provides the linkage between the CH3 domain and the characterised VH domain of known binding specificity. The hinge/VH are subsequently introduced into the PAC
 5 containing the human VH locus (figure 1) by standard homologous recombination in bacteria.

The Novel PAC is subsequently grown and the locus isolated by standard restriction digestion (see Janssens R, Dekker S, Hendriks RW, Panayotou G, van Remoortere A, San JK, Grosveld F, Drabek D. Generation of heavy-chain-only antibodies in mice.

Proc Natl Acad Sci U S A. 2006;103:15130-5) and injected into fertilized mouse eggs to
 10 generate transgenic mice containing the locus containing tetravalent bi-specific VH homodimers. Tetravalent bispecific antibodies are generated in response to antigen challenge. The N terminal VH binding domains of these homodimers are novel and specifically bind the antigen used for immunisation, The C terminal VH binding domain will bind a predetermined antigen.

15 Thus if the C-terminal binding domain recognizes human CR1, the tetravalent VH binding complex will bind to red blood cells. If the N-terminal VH binding domain is generated in response to a pathogen, then the tetravalent VH binding complex when introduced in vivo will bind CR1 and pathogens simultaneously, and the antigen will be transported efficiently to the liver for destruction (Repik A, Pincus SE, Ghiran I, Nicholson-Weller A, Asher DR,
 20 Cerny AM, Casey LS, Jones SM, Jones SN, Mohamed N, Klickstein LB, Spitalny G, Finberg RW. A transgenic mouse model for studying the clearance of blood-borne pathogens via human complement receptor 1 (CR1). Clin Exp Immunol. 2005;140:230-40).

Obviously the C terminal VH binding domain could be any predetermined antigen specific VH binding domain which recognises a target cell (eg T or B-cell), a cell surface receptor (eg
 25 EGFR or Her-2 on a tumour surface) or any target antigen.

EXAMPLE 2

Generation of a germline locus to generate tetravalent bi-specific VH homodimers containing at the C-terminus an additional marker protein. In this example a C terminal marker protein is a GFP (Green Fluorescent Protein, Heim R, Cubitt AB, Tsien RY. Improved green
 30 fluorescence. Nature. 1995; 23;373:663-4) is added to the additional binding domain. The

generation of such a locus is identical to that described in example 1 with the exception that a gene encoding a marker protein eg GFP is placed between the CH3 domain and the C-terminal hinge and VH binding domain (Figure 3). The GFP gene would first be cloned onto the 5' end of the hinge by conventional means. The cloning into the vector would use oligonucleotides with the same homology to the locus but would be different at their 3' end to match the GFP marker gene. The resulting mice will produce specific VH based homodimers in response to immunization that carry a fluorescent marker which can be used for diagnostic purposes, protein tracking, gene expression assays or screening purposes. Obviously the GFP coding gene can be replaced by a large number of other genes coding for fluorescent or other types of markers/protein tags.

EXAMPLE 3

Generation of a germline locus to generate tetravalent-valent bi-specific VH homodimers containing at the C-terminus an additional enzyme activity. In this example the C terminal enzyme is the prodrug converting enzyme nitroreductase.

The generation of such a locus is identical to that described in example 1 with the exception that the gene coding for nitroreductase (see Drabek D, Guy J, Craig R, Grosveld F. The expression of bacterial nitroreductase in transgenic mice results in specific cell killing by the prodrug CB1954. *Gene Ther.* 1997;4:93-100) is placed between the CH3 domain and C-terminal hinge and the VH binding domain (Figure 4). The NTR gene would first be cloned onto the 5' end of the hinge by conventional means. The cloning into the vector would use oligonucleotides with the same homology to the locus but would be different at their 3' end to match the NTR gene. The resulting mice will produce specific VH based homodimers in response to immunization that carry a nitroreductase gene that will convert the prodrug CB1954 into a cytotoxic DNA interstrand cross-linking agent. In this particular embodiment both N- and C-terminal VH binding domains might bind surface antigen present on tumours such as EGRF and Her-2. Such VH binding molecules can be used for specific cell type killing such as in anti-tumour treatment. Obviously the C terminal additional protein could be any protein with desired functionality an enzyme, e.g. proteases, phosphatase, kinase, or carrier protein eg albumin.

EXAMPLE 4

This example shows a further extension of the principles shown in example 1. Instead of adding one extra hinge and binding domain at the end of the CH3 exon, multiple domains are added either downstream of the J regions and/or downstream of the CH3 exon.

- 5 In the first step the hinge and one or more binding domains of known binding specificity are cloned into a recombination vector by standard methods using the oligonucleotides similar to the principle described in Figure 2 and PCR amplification followed by standard cloning. The single or multiple domains can be placed downstream of the J region and/or downstream of the CH3 region as described in example 1. This provides the linkage between the J and/or
- 10 CH3 domain and the binding domains of known binding specificity (or other effector regions). The new domains are subsequently introduced into the PAC containing the human VH locus (figure 1) by standard homologous recombination in bacteria. The resulting locus would be injected into transgenic mice to generate multivalent multi-specific VH homodimers

CLAIMS

1. A method for the production of a homodimeric VH binding complex in a transgenic non-human mammal in response to antigen challenge, comprising the step of providing one or more heterologous VH binding complex loci in that mammal, wherein each VH binding
5 complex locus is defined by the formula 5'-A-B-C-D-3' wherein: A comprises one or more V gene segments, one or more D gene segments and one or more J gene segments; B is optional and encodes one or more characterised binding domains of known specificity; C encodes a CH2-CH3 dimerisation domain; and D encodes one or more characterised binding domains of known specificity.
- 10 2. The method of claim 1, which produces a homodimeric VH binding complex which is tetravalent and bispecific.
3. The method of claim 1 which produces a homodimeric VH binding complex which is multivalent and bispecific or multispecific.
4. The method of any one of claims 1 to 3, which produces a homodimeric VH binding
15 complex which has a hinge domain or a flexible hinge-like peptidic linker linking any one or more of the adjacent pairs of domains.
5. The method of any one of claims 1 to 4, wherein C further comprises a CH4 exon.
6. The method of any one of claims 1 to 5, wherein the V, D and J gene segments, the CH2-CH3 dimerisation domain-encoding sequence and the sequences encoding the other
20 binding domains in any one VH binding complex locus are naturally derived or engineered genes from an organism of the same species.
7. The method of any one of claims 1 to 5, wherein the V, D and J gene segments, the CH2-CH3 dimerisation domain-encoding sequence and the sequences encoding the other
25 binding domains in any one VH binding complex locus are naturally derived or engineered genes from an organism of different species.
8. The method of claim 6, wherein the V, D and J gene segments, the CH2-CH3 dimerisation domain-encoding sequence and the sequences encoding the other binding domains are naturally derived or engineered human genes.

9. The method of any one of the previous claims, wherein the one or more VH binding complex loci comprise any number or combination of the 39 functional human V gene segments and engineered variants thereof with improved solubility properties distributed across one or more VH binding complex loci.
- 5 10. The method of claim 1, wherein each VH binding complex locus comprises one or more natural or engineered human V gene segments, twenty-five functional human D gene segments and 6 human J gene segments.
11. The method of any one of claims 1 to 10 wherein the CH2-CH3 dimerisation domain is derived from IgG, IgD or IgE.
- 10 12. The method of any one of claims 1 to 10, wherein the CH2-CH3 (-CH4) dimerisation domain is derived from IgM or IgA.
13. The method of any one of the previous claims, wherein the predetermined binding domains B and D have been engineered so as to minimise somatic mutation as a result of AIDS enzyme activity.
- 15 14. A method of any one of the previous claims, wherein the dimerisation domain C also comprises a CH1 domain, and the resultant VH binding complex locus is expressed in a mammalian host background lacking host immunoglobulin light chain functionality, and optionally lacking host immunoglobulin heavy chain functionality.
- 20 15. A transgenic non-human mammal as defined in any one of the preceding claims comprising one or more identical or different heterologous homodimeric VH binding complex loci.
16. A method for the production of antigen-specific homodimeric VH binding complex by:
- immunising a transgenic non-human mammal according to claim 15 with an antigen;
- 25 generating B-cell hybridomas;
- selecting cells expressing antigen-specific homodimeric VH binding complexes; and

isolating and characterising antigen-specific homodimeric VH binding complexes secreted by hybridoma cells.

17. A method for the production of antigen-specific VH binding domains from homodimeric VH binding complexes by immunising a transgenic non-human mammal
5 according to claim 15 with an antigen and then either:

(i) generating B-cell hybridomas,

selecting for cells expressing antigen-specific VH binding complexes,

isolating mRNA from said hybridomas, and

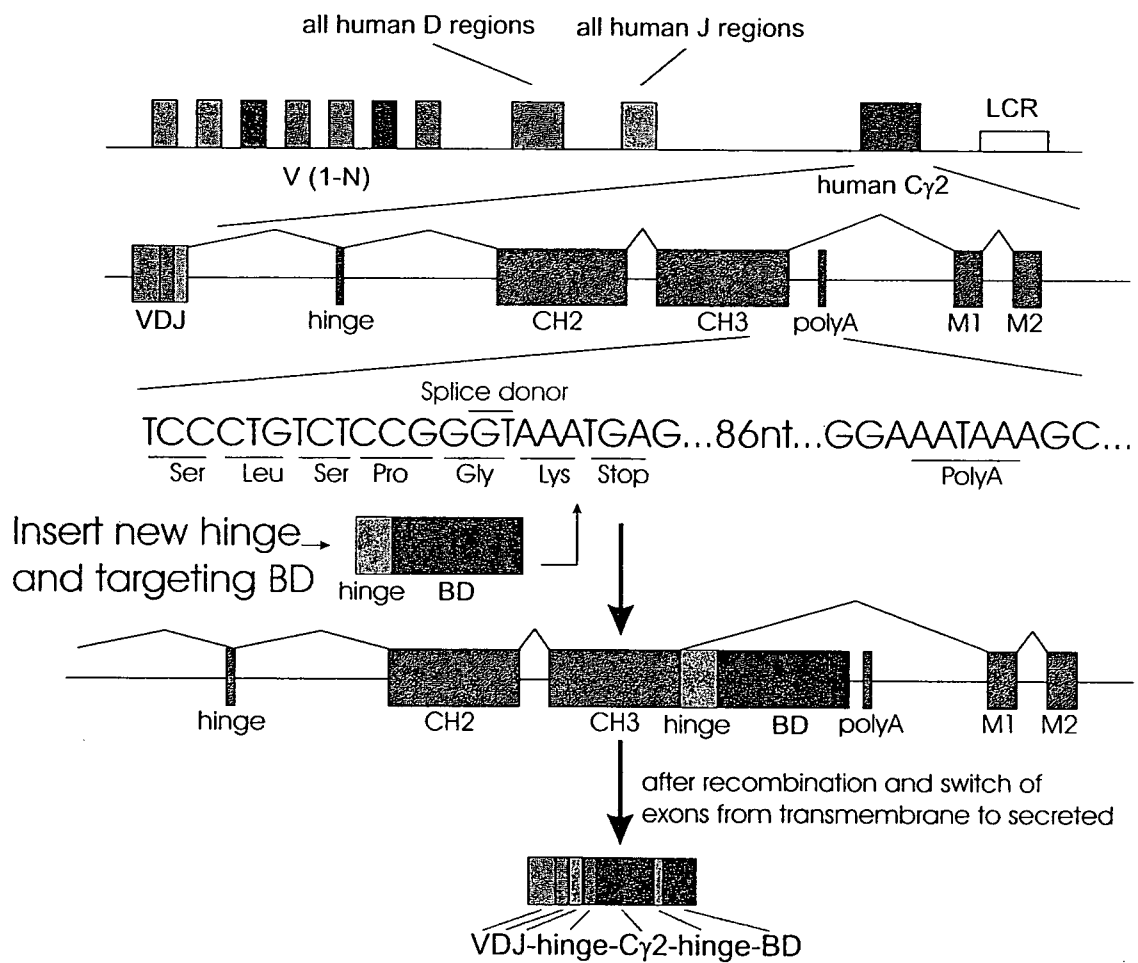
cloning and sequencing the amino terminal VH binding domain;

10 or

(ii) isolating mRNA from B-cells (including spleen),

cloning 5' VH domains into display libraries,

selecting for antigen binding and sequencing antigen-specific VH binding domains.

FIG. 1

Oligo's (1 +2 and 3+4) are annealed to form linkers

- 1: LINK5'BDhigG2h3F 5'GTACCGCATGAGGCTCTGCACAACCACTACACACAGAGAG CCTCTCCCTGTCTCCGGGTAA
- 2 : LINK5'BDhigG2h3R 5'AGCTTTACCCGGAGACAGGAGAGGGCTCTTCTGTGTGTAG TGGTTGTGCAGAGCCTCATGCCG
- 3 : LINK3'BDhigG2ER1F 5'AATTCTGAGTGCCACGGCCGGCAAAGCCCCCGCTCCCCAGGCTCTCGGGGTGCGCGGAGGATGCG
- 4 : LINK3'BDhigG2ER1R 5'GTACCGCATCTCTCGCGGACCCCGAGAGCCTGGGAGCG GGGGCTTGCCGGCCGTGGCACTCAG

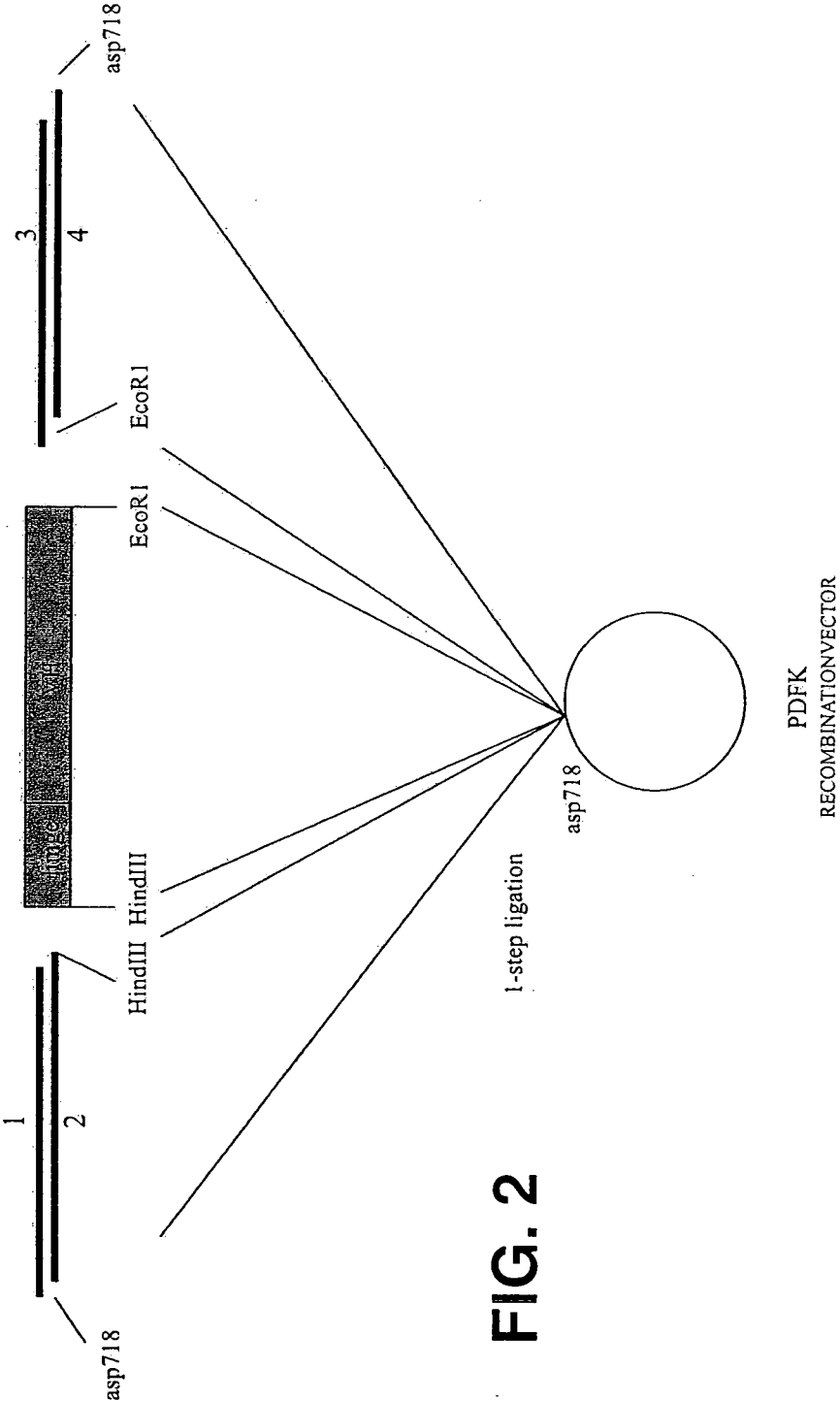


FIG. 2

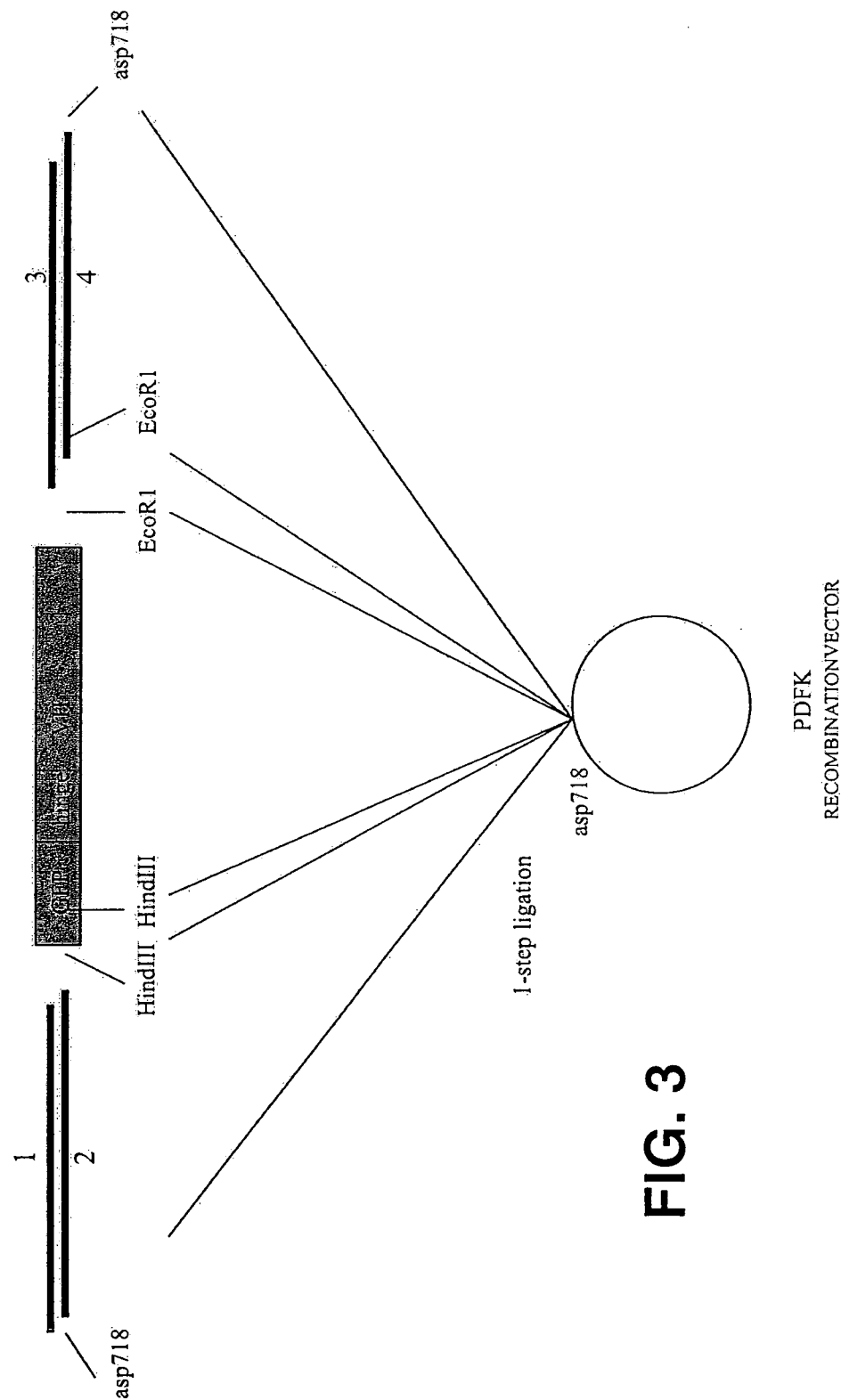


FIG. 3

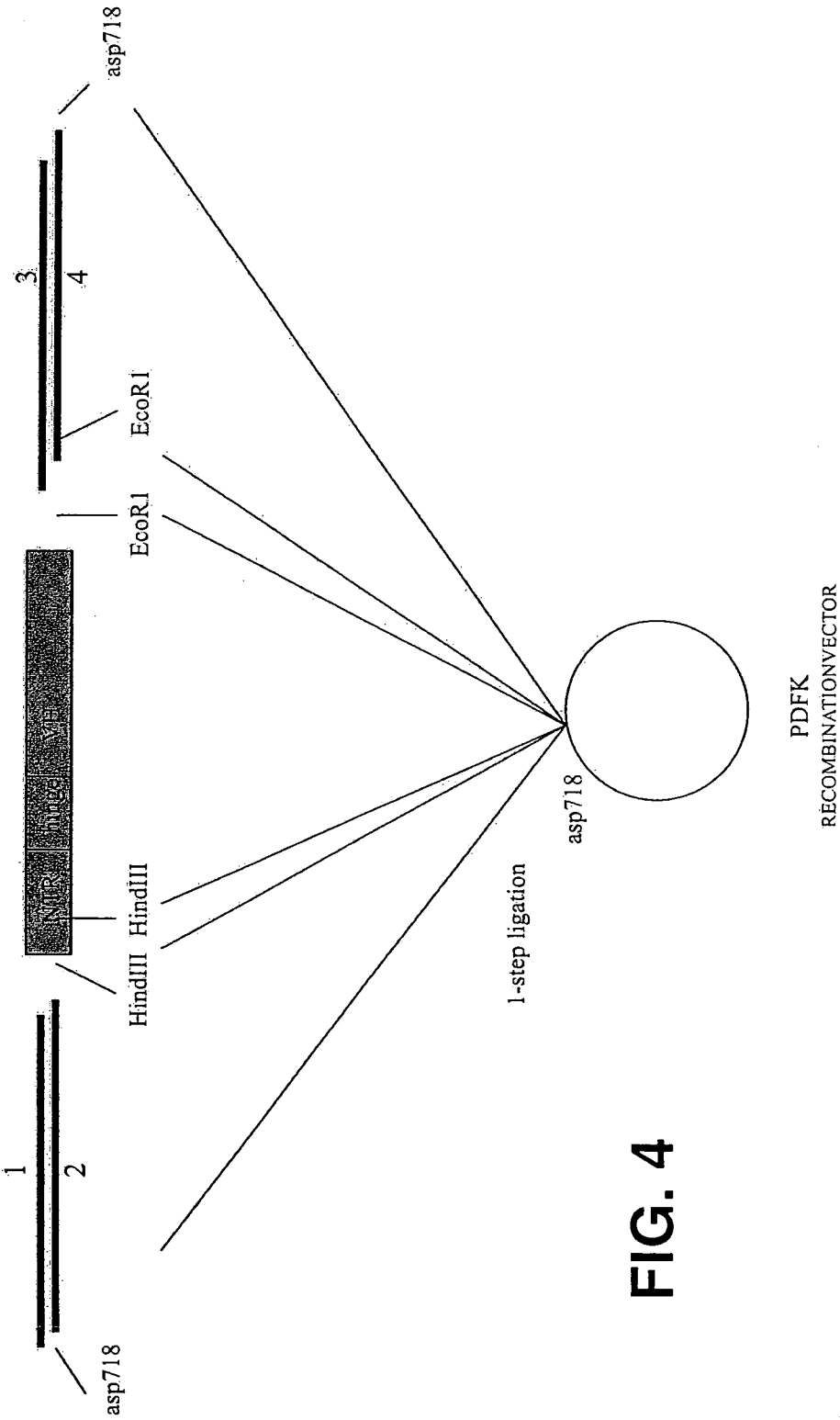


FIG. 4

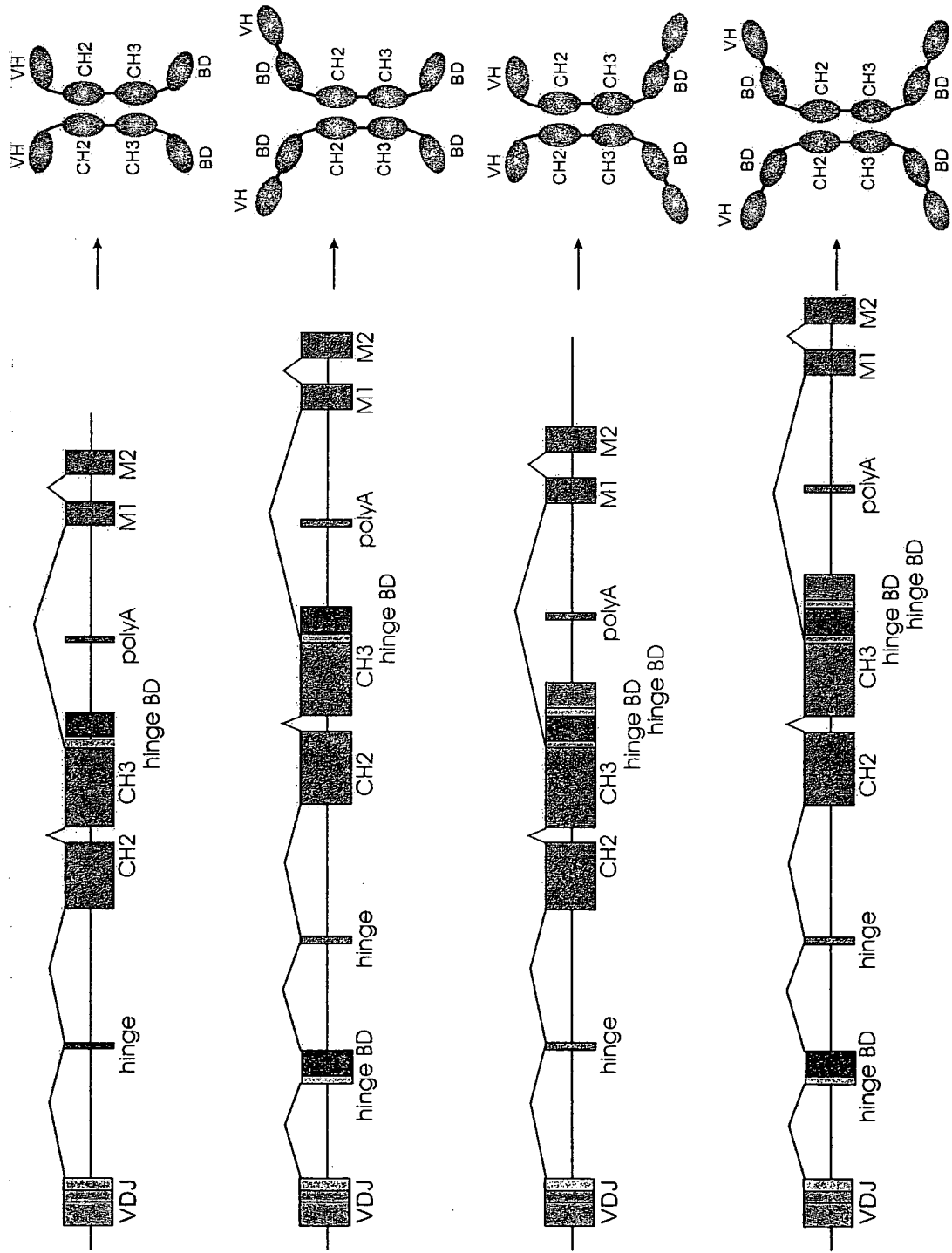


FIG. 5