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Description

Field of the Invention

[0001] The present invention relates to a method for the production of heavy chain-only antibodies in transgenic rats.

Background to the Invention

[0002] Monoclonal antibodies or variants thereof 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 monoclonal antibody products recognise and bind a single, well-defined epitope on the target ligand (eg TNFα). Manufacture of human monoclonal antibodies for therapy remains dependent on mammalian cell culture. The assembly of a complex consisting of two heavy chains and two light chains (the H_2L_2 complex) and subsequent post-translational glycosylation processes preclude the use of bacterial systems. Production costs and capital costs for antibody manufacture by mammalian cell culture are high and threaten to limit the potential of antibody based therapies in the absence of acceptable alternatives. A variety of transgenic organisms are capable of expressing fully functional antibodies. These include plants, insects, chickens, goats and cattle but none as yet has been used to manufacture marketed therapeutic products.

[0003] Functional antibody fragments can be manufactured in *E. coli* but the product generally has low serum stability unless pegylated during the manufacturing process.

[0004] Bispecific antibody complexes are engineered Ig-based molecules capable of binding two different epitopes on the either the same or different antigens. Bispecific 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 Spriel 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 Spriel 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).

[0005] Manufacturing issues are compounded where a bi-specific antibody product is based on two or more H_2L_2 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).

[0006] To address this issue, a number of strategies have been developed for the production in mammalian cells of full length bispecific IgG formats (BsIgG) which retain heavy chain effector function. BsIgGs require engineered "knob and hole" heavy chains to prevent heterodimer formation and utilise identical L-chains to avoid L-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 (Tacken et al., (2004) J. Immunol., 172, 4934-4940).

[0007] 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 bispecific 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 L-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).

[0008] Recently, the BsAb concept has been extended to encompass Di-diabodies, tetravalent bispecific antibodies where the V_H and V_L 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).

[0009] The structure of immunoglobulins is well known in the art. Most natural immunoglobulins comprise 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 disulphide bond close to the hinge domain.

[0010] When an Ig molecule is correctly folded, each chain folds into a number of distinct globular domains joined by

a more linear polypeptide sequence. For example, the light chain folds into a variable (V_L) and a constant (C_L) domain. Heavy chains have a single variable domain V_H, adjacent the variable domain of the light chain, a first constant domain, a hinge domain and two or three further constant domains. Interaction of the heavy (V_H) and light (V_L) chain variable domains results in the formation of an antigen binding region (Fv). Generally, both V_H and V_L are required for antigen binding, although heavy chain dimers and amino-terminal fragments have been shown to retain activity in the absence of light chain (Jaton et al., (1968) Biochemistry, 7, 4185-4195).

[0011] 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 molecular level showed that mutations and deletions at the level of the genome could result in inappropriate expression of the heavy chain C_H1 domain, giving rise to the expression of heavy chain-only antibody lacking the ability to bind light chain (see Hendershot et al., (1987) J. Cell Biol., 104, 761-767; Brandt et al., (1984) Mol. Cell. Biol., 4, 1270-1277).

[0012] Separate studies on isolated human V_H domains derived from phage libraries demonstrated antigen-specific binding of V_H domains but these V_H domains proved to be of low solubility. Furthermore, it was suggested that the selection of human V_H domains with specific binding characteristics displayed on phage arrays could form the building blocks for engineered antibodies (Ward et al., (1989) Nature, 341, 544-546).

[0013] Studies using other vertebrate species have shown that camelids, as a result of natural gene mutations, produce functional IgG2 and IgG3 heavy chain-only dimers which are unable to bind light chain due to the absence of the C_H1 light chain-binding region (Hamers-Casterman et al., (1993) Nature, 363, 446-448) and that species such as shark produce a heavy chain-only-like binding protein family, probably related to the mammalian T-cell receptor or immunoglobulin light chain (Stanfield et al., (2004) Science, 305, 1770-1773).

[0014] A characterising feature of the camelid heavy chain-only antibody is the camelid V_H domain, which provides improved solubility relative to the human V_H domain. Human V_H may be engineered for improved solubility characteristics (see Davies and Riechmann, (1996) Protein Eng., 9 (6), 531-537; Lutz and Muyldermans, (1999) J. Immuno. Methods, 231, 25-38) or solubility maybe be acquired by natural selection *in vivo* (see Tanha et al., (2001) J. Biol. Chem., 276, 24774-24780). However, where V_H 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).

[0015] Camelid V_H antibodies are also characterised by a modified CDR3 loop. This CDR3 loop is, on average, longer than those found in non-camelid antibodies and is a feature considered to be a major influence on overall antigen affinity and specificity, which compensates for the absence of a V_L 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).

[0016] Recent structural studies on camelid antibody suggests that antibody diversity is largely driven by *in vivo* maturation processes with dependency on V(D)J recombination events and somatic mutation, (De Genst et al,. (2005) J. Biol. Chem., 280 (14), 14114-14121).

[0017] Recently, methods for the production of heavy-chain-only antibodies in transgenic mammals have been developed (see WO02/085945 and WO02/085944). Functional heavy chain-only antibody of potentially any class (IgM, IgG, IgD, IgA or IgE) and derived from any mammal (including man) can be produced from transgenic mammals (preferably mice) as a result of antigen challenge.

[0018] The normal immunoglobulin heavy chain locus comprises a plurality of V gene segments, a number of D gene segments and a number of J gene segments. Each V gene segment encodes from the N terminal almost to the C terminal of a V domain. The C terminal end of each V domain is encoded by a D gene segment and a J gene segment. VDJ rearrangement in B-cells followed by affinity maturation provides V_H binding domains which then, with V_L binding domains, form an antigen recognition or binding site.

[0019] Interaction of the heavy and light chains is facilitated by the C_H1 region of the heavy chain and the κ or λ region of the light chain.

[0020] For the production of heavy chain-only antibody, the heavy chain locus in the germline comprises gene segments encoding some or all of the possible constant regions. During maturation, a re-arranged V_H binding domain is spliced onto the C_H2 constant region-encoding segment, to provide a re-arranged gene encoding a heavy chain which lacks a C_H1 domain and is therefore unable to associate with an immunoglobulin light chain.

[0021] Heavy chain-only monoclonal antibodies can be recovered from B-cells of the spleen by standard cloning technology or recovered from B-cell mRNA by phage display technology (Ward et al., (1989) Nature, 341, 544-546). Heavy chain-only antibodies derived from camelids or transgenic animals are of high affinity. Sequence analysis of normal H_2L_2 tetramers demonstrates that diversity results primarily from a combination of VDJ rearrangement and somatic hypermutation (Xu and Davies, (2000) Immunity, 13, 37-45). Sequence analysis of expressed heavy chain-only mRNA, whether produced in camelids or transgenic animals, supports this observation (De Genst et al., (2005) J. Biol. Chem., 280, 14114-14121).

[0022] An important and common feature of natural camelid and human V_H regions is that each region binds as a monomer with no dependency on dimerisation with a V_L region for optimal solubility and binding affinity. These features have previously been recognised as particularly suited to the production of blocking agents and tissue penetration agents.

[0023] Homo- or hetero-dimers can also be generated by enzymatic cleavage of heavy chain-only antibodies or by synthetic routes (Jaton et al., (1968) Biochemistry, 7, 4185-4195 and US2003/0058074 A1). However the benefits of a monomeric antibody binding domain have yet to be used to advantage in design of multimeric proteins as reagents, therapeutics and diagnostics.

[0024] Human V_H or camelid V_{HH} produced by phage display technology lacks the advantage of improved characteristics as a result of somatic mutations and the additional diversity provided by D and J region recombination in the CDR3 region of the normal antibody binding site (Xu and Davies, (2000) Immunity, 13, 37-45). Camelid V_{HH}, whilst showing benefits in solubility relative to human V_H, is antigenic in man and must be generated by immunisation of camelids or by phage display technology.

[0025] The incorporation of V_H binding domains has clear advantage over the use of scFvs which must be engineered from V_H and V_L domains with the associated potential of loss of specificity and avidity. V_H binding domains derived from related gene families such as T-cell receptors or the shark immunogloblin family also provide alternatives to scFv for the generation of bi- or multi-specific binding molecules. Other naturally occurring binding proteins and domains thereof including, for example, soluble receptor fragments may also be used.

[0026] Antibody classes differ in their physiological function. For example, IgG plays a dominant role in a mature immune response. IgM is involved in complement fixing and agglutination. IgA is the major class of Ig in secretions - tears, saliva, colostrum, mucus - and thus plays a role in local immunity. The inclusion of class-specific heavy chain constant regions when engineering multivalent binding complexes provides the therapeutic benefits of effector function *in vivo* dependent on the functionality required. Engineering of individual effector regions 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). It seems likely that the optimal production and selection of heavy chain-only antibodies comprising high affinity V_H binding domains (whether of human or camelid or other origin) will benefit from alternative approaches to those dependent on selection from randomised phage libraries which do not facilitate *in vivo* recombination and affinity maturation.

[0027] Thus, the inclusion of IgA constant region functionality would provide improved mucosal function against pathogens (Leher et al., (1999) Exp. Eye. Res., 69, 75-84), whilst the presence of IgG1 constant region functionality provides enhanced serum stability *in vivo.* The presence of heavy chain C_H2 and C_H3 constant domains provides the basis for stable dimerisation as seen in natural antibodies, and provides recognition sites for post-translational glycosylation. The presence of C_H2 and C_H3 also allows for secondary antibody recognition when bispecific and multivalent complexes are used as reagents and diagnostics.

[0028] Isolated, pre-rearranged camelid heavy chain-only variable region sequences have previously been cloned in front of a hinge region and human IgG1 effector domain, inserted into vectors and expressed in COS cells to generate antibody. The antibodies expressed in this *in vitro* environment have already undergone the processes of class (isotype) switching and affinity maturation (hypermutation) *in vivo* in the camel and can bind to antigen (Riechmann and Muyldermans, (1999) J. Immunol. Methods, 231, 25-38).

[0029] WO 2004/049794 describes single chain antibodies, a transgenic mouse and single chain antibodies produced by such a transgenic mouse.

[0030] There remains a need in the art to generate a functional repertoire of class specific human heavy chain-only antibodies and functional V_H heavy chain-only binding domains which retain maximum antigen-binding potential for use in diverse clinical, industrial and research applications.

Brief Summary of the Invention

[0031] The present invention provides a method for the production of a V_H heavy chain-only antibody comprising:

(a) immunising a transgenic rat expressing a heterologous V_H heavy chain locus with an antigen, wherein:

(i) the V_H heavy chain locus comprises a variable region comprising at least one naturally-occurring V_H gene segment, at least one D gene segment, at least one J gene segment and at least one heavy chain constant region, and wherein the V, D and J gene segments are derived from a human;
(ii) each constant region does not encode a C_H1 domain;
(iii) a V_H gene segment, a D gene segment and a J gene segment are capable of recombining to form a VDJ coding sequence;
(iv) the recombined V_H heavy chain locus, when expressed, is capable of forming a soluble, heavy chain-only antibody comprising a soluble, antigen-specific V_H binding domain and a constant effector region devoid of a C_H1 domain;

(b) isolating a nucleic acid sequence encoding the V_H heavy chain-only antibody from antibody producing cells; and
(c) producing the V_H heavy chain-only antibody using recombinant DNA techniques.

[0032] In certain embodiments, the method of the invention further comprises cloning a V_H locus encoding the V_H binding domain of the V_H heavy chain-only antibody and expressing the V_H binding domain in a bacterial, yeast, mammalian or alternative expression system.

[0033] Preferably, said transgenic rat has been engineered to have a reduced capacity to produce antibodies which include light chains.

[0034] Preferably, immunoglobulin heavy chain loci endogenous to the rat are deleted or silenced. Preferably, the V_H heavy chain locus comprises more than one V gene segment, more than one D gene segment and more than one J gene segment.
Preferably, the V gene segment has been selected to show improved solubility characteristics.

[0035] The heavy chain constant region of the heavy chain locus may comprise a $C\alpha_1$ and/or a $C\alpha_2$, a $C\varepsilon$, a $C\delta$, a $C\gamma$ and/or a $C\mu$ heavy chain constant region gene. Furthermore, the heavy chain constant region of the heavy chain locus may comprise more than one of the following heavy chain constant regions: $C\alpha_1$, $C\alpha_2$, $C\varepsilon$, $C\delta$, $C\gamma$ $C\mu$.

[0036] The V_H heavy chain locus comprises a variable region comprising at least one human V gene segment, at least one human D segment and at least one human J segment wherein a V gene, a D gene and a J gene segment are capable of recombining to form a VDJ coding sequence. The heavy chain locus preferably comprises twenty or more D gene segments and/or five or more J gene segments. The CDR3 loop is derived using human D and J gene segments.

[0037] The V_H heavy chain locus may also comprise a recombination sequence (rss) capable of recombining a J gene segment directly with a heavy chain constant region gene.

[0038] The heavy chain constant region of the heterologous heavy chain locus is of human origin or vertebrate origin e.g. of camelid origin. Alternatively the constant region may not be of immunoglobulin heavy chain origin.

[0039] The method of the invention results in essentially normal B-cell maturation. The V_H binding domain may lack an extended camelid-like CDR3 loop.

[0040] The invention also provides a method of production and selection of heavy chain-only antibodies comprising the steps of:

(a) injecting an antigen into the transgenic mammal as described herein;

b) isolating a cell or tissue expressing an antigen-specific, heavy chain-only antibody of interest; and

c) producing a hybridoma from the cell or tissue of step (b) and

d) optionally cloning the heavy chain-only antibody mRNA from said hybridoma for subsequent production in a heterologous expression system such as a mammalian, plant, insect, microbial, fungal or alternative system.

[0041] V_H binding domains may then be produced by identifying and isolating an antigen-specific V_H domain from the cloned mRNA of step c).

[0042] V_H binding domains may also be produced by:

(a) injecting an antigen into the transgenic mammal described herein;

b) isolating a cell or tissue expressing an antigen-specific, heavy chain-only antibody of interest;

c) cloning the V_H locus from mRNA derived from the isolated cell or tissue;

d) displaying the encoded protein using a phage or similar library;

e) identifying antigen-specific V_H domain(s); and

f) expressing the V_H domain(s) alone or as a fusion protein in bacterial, yeast or alternative expression systems.

DETAILED DESCRIPTION OF THE INVENTION

[0043] The present inventors have overcome the limitations of the prior art and shown that transgenic animals, in particular mice, can be generated using "micro loci" to produce class-specific, heavy chain-only antibodies, or a mixture of different classes of heavy chain-only antibodies which are secreted by plasma or B cells. These can then be used

either to generate a reliable supply of class-specific, heavy chain-only antibody using established hybridoma technology or as a source of functional V_H heavy chain-only binding domains, preferably a soluble V_H heavy chain-only binding domains of human origin, which are free of effector functions but which retain binding function.

[0044] Heavy chain-only antibodies that can be generated by the method of the invention show high binding affinity, resulting from V, D and J gene segment rearrangements and somatic mutations, 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 C_H1 domain has been eliminated from all antibody classes present in the recombinant locus). B-cell maturation and the secretion of assembled dimers (eg IgG) or multimers (eg IgM) has no dependency on the presence or expression of light chain genes.

[0045] Nucleotide sequence analysis of antigen-specific mRNA encoding an antigen-specific heavy chain isolated from hybridomas derived from transgenic mice has demonstrated that heavy chain antibody diversity is primarily a function of VDJ recombination. Furthermore, the present inventors have shown that antibody diversity is generated in the CDR3 region of the functional antigen-binding domain of the heavy chain-only antibody with a more limited contribution from somatic mutations in the V_H domains. Using the methods described herein, functional V_H domains can be cloned and expressed in bacterial systems to generate V_H binding domains with full retention of antigen binding, specificity and affinity.

[0046] It is shown that transgenic mice can be programmed to produce preferred classes of heavy chain-only antibody in response to antigen challenge, eg only IgG as opposed to only IgM or, for example, mixtures of IgA, IgG and IgM.

[0047] The inventors have previously described (see WO02/085945 and WO02/085944) the generation of transgenic mice expressing a minimal human IgG heavy chain constant region locus devoid of the C_H1 exon and linked by human D and J segments with two llama VHH genes. These produce functional, high affinity, antigen-specific IgG heavy chain-only antibody when challenged with antigen. Mixtures of heavy chain-only antibody classes (IgM and IgG) can be obtained by class switching *in vivo* through utilisation of gene constructs incorporating heavy chain constant regions in tandem (provided that all constant region genes lack a C_H1 domain and, when present, a C_H4 domain).

[0048] The improvements described herein show that a mouse constructed with the same IgG constant region locus linked by human D and J segments with two llama V_{HH} genes and a human IgM constant region locus devoid of a C_H1 exon linked by the same human D and J gene segments with two llama V_{HH} genes, also produces high molecular weight (multimeric) IgM heavy chain-only antibody and IgG (dimer) heavy chain-only antibody. Surprisingly, essentially normal B-cell maturation and antibody production is dependent on the complete absence of C_H1 sequences from each heavy chain constant region present in the transgenic locus. Moreover, there is no requirement for the removal of the C_H4 exon if present.

[0049] Thus, for example, a transgenic animal carrying a human IgM heavy chain locus with a functional C_H1 exon linked by the same human D and J gene segments to two llama V gene segments, and IgG constant heavy chain region locus devoid of the C_H1 exon linked by the same human D and J gene segments to two llama V gene segments, produces very low levels of heavy chain-only antibody and shows no evidence for B-cell maturation.

[0050] Other effector domains, including the C_H4 domain, may be incorporated or not, as desired, to introduce to, or eliminate from, the resultant heavy chain-only antibody, effector features.

[0051] The inventors have found that productive expression of antibody (ie B-cell maturation) can result from the use of any V gene segment present in the construct. Isolation and sequencing of antibody mRNA derived from B-cells shows that D and J gene segment recombination occurs to generate CDR3 diversity. Sequence comparison of resultant V_H domains reveals somatic mutations, indicating that affinity maturation events have occurred in the recombined D and J gene segments and also in the V_H domain of the resultant expressed antibody mRNA.

[0052] Preferred constructs incorporate V gene segments selected for improved solubility and linked to a D and J chain cluster for recombination and CDR3 generation. Preferably, the VDJ sequences are linked to constant effector domain(s) of choice in tandem, each devoid of a C_H1 exon.

[0053] The resultant V_H domains may not comprise an enlarged camelid-like CDR3 loop. This results in a V_H domain exhibiting CDR3 diversity and affinity maturation operationally linked to an effector constant region. The latter ensures functional secretion and optionally assembly in the parent rat.

[0054] These observations have important implications for the improved and simplified engineering of class-specific, heavy chain-only antibodies and the derivation of high affinity, soluble V_H domains which incorporate affinity maturation via somatic mutation. Incorporation of select heavy chain constant region effector functions (devoid of C_H1) or mixtures thereof permits the production of any class of heavy chain-only antibodies or any mixture of heavy chain-only antibodies without the requirement of additional antibody engineering. V_H domains can be expressed alone in bacterial or other micro-organism systems or as functional heavy chain-only antibody incorporating effector domains secreted by hybridomas or transfected cells in culture. Antibodies and V_H binding domains of human origin have wide ranging applications in the field of healthcare as medicines, diagnostics and reagents, with parallel agricultural, environmental and industrial applications.

[0055] Heavy chain effector molecules may be engineered to be free of functional domains, for example the carboxy-

terminal C_H4 domains, provided that engineering does not affect secretory mechanisms preventing cell surface assembly and consequently B-cell maturation. The C_H1 exons alone are deleted from the heterologous locus or are absent from the locus. Additional features maybe engineered into the locus, for example to improve glycosylation, or add function.

[0056] Preferably, the heterologous locus, when expressed, is capable of forming functional IgA, IgE, IgG, IgD or IgM molecules or isotypes thereof. Individual antibody classes or mixtures of antibody classes or isotypes thereof may also be produced.

[0057] Accordingly, the heterologous heavy chain locus is designed to produce preferred classes or mixtures of heavy chain-only antibody depending on the antibody class(es) required, with essentially normal B-cell maturation. The use of human V, D and J gene segments comprising V gene segments randomly selected, or selected for enhanced solubility, will produce functional human heavy chain-only antibodies.

[0058] Antibodies obtained in the method of the invention have the advantage over those of the prior art in that they are of substantially any single or known class and of human origin. Antibodies are of high affinity resulting from a combination of VDJ recombination and affinity maturation *in vivo.* Antibodies and fragments thereof may be may be isolated, characterised and manufactured using well-established methods known to those skilled in the art.

The Heterologous Heavy Chain Locus

[0059] 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.

[0060] A "V_H heavy chain locus" in the context of the present invention relates to a minimal micro-locus encoding a V_H domain comprising one or more V gene segments, one or more D gene segments and one or more J gene segments, operationally linked to one or more heavy chain effector regions (each devoid of a C_H1 domain). Preferably, the primary source of antibody repertoire variability is the CDR3 region formed by the selection of D and J gene segments by the V-D and D-J junctions.

[0061] The advantage of the present invention is that antibody repertoire and diversity obtained in the rearranged V_H gene sequences can be maximised through the use of multiple D and J gene segments. Subsequent somatic mutation is achieved whilst using a minimal locus (micro-locus) without the need for a large number of V gene segments or the V_L and Lc (light chain) immunoglobulin loci.

[0062] Preferably, the V_H heavy chain locus comprises from two to five V (2, 3, 4 or 5) gene segments.

[0063] The V gene segments are of human origin, optionally selected for improved solubility.

[0064] Preferably, the V_H heavy chain locus comprises from two to forty (2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 30 or 40) or more D gene segments (normally 25 functional human D gene segments).

[0065] Preferably, the V_H heavy chain locus comprises from two to twenty (2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18 or 20) or more J gene segments (normally 6 human J gene segments).

[0066] Preferably, the V_H heavy chain locus comprises two or more human V gene segments, twenty-five functional human D gene segments and 6 human J gene segments.

[0067] The term 'V gene segment' encompasses a naturally occurring V gene segment derived from a human, which have optionally been selected for improved characteristics, such as solubility.

[0068] Preferred methods of improving solubility of a V_H domain incorporate rational, as opposed to only random, means and are exemplified in Davies and Reichmann, (1996) Protein Eng., 9 (6), 531-537 and Riechmann and Muyldermans, (1999) J. Immunol. Methods, 231, 25-38. Natural selection can also occur *in vivo* through affinity maturation and the incorporation of favourable mutations in the V_H gene following VDJ re-arrangement.

[0069] The V gene segment must be capable of recombining with a D gene segment, a J gene segment and a heavy chain constant (effector) region (which may comprise several exons but excludes a C_H1 exon) according to the present invention to generate a V_H heavy chain-only antibody when the nucleic acid is expressed.

[0070] Thus V_H coding sequences may be derived from a naturally occurring source or they may be synthesised using methods familiar to those skilled in the art.

[0071] A "V_H 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. The V_H domain as used herein remains in solution and is active in a physiological medium without the need for any other factor to maintain solubility. Preferably, the ability of the soluble V_H domain to bind antigen has been improved by 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 V_H domain is able to bind antigen as a monomer and, when combined with effector constant regions, may be produced in monospecific, bi-specific, multi-specific, bi-valent or multivalent forms, dependent on the choice and engineering of the effector molecules used (eg IgG, IgA IgM etc.) or alternative mechanisms of dimerisation and multimerisation. Any likelihood of binding with a V_L domain when expressed as part of a soluble heavy chain-only antibody complex has been eliminated by removal of the C_H1 exon (see Sitia et al., (1990) Cell, 60, 781-790). The V_H domain alone can also be engineered with diverse protein domains to produce fusion proteins for targeted therapeutic and

diagnostic purpose, for example with toxins, enzymes and imaging agents.

[0072] The heterologous heavy chain locus comprises a region of DNA encoding a heavy chain constant region providing effector functions *in vivo* (eg IgG, IgM, IgA, IgE, IgD or isotypes thereof).

The heavy chain constant region

[0073] Operationally, a heavy chain constant region is 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. Preferably the heavy chain constant region is derived from an immunoglobulin locus.

[0074] Each heavy chain constant region essentially comprises at least one heavy chain constant region gene, which is expressed without a functional C_H1 domain so that generation of heavy chain-only antibody can occur. Each heavy chain constant region may also comprise one or more additional heavy chain constant region exons, which are selected from the group consisting of $C\delta$, $C\gamma_{1\text{-}4}$, $C\mu$, $C\varepsilon$, and $C\alpha_{1\text{-}2}$ with the proviso that the additional heavy chain constant region genes also do not express a functional C_H1 domain. The heavy chain constant region gene segments are selected depending on the preferred class or mixture of antibody classes required. Optionally, the heterologous heavy chain locus is $C\mu$- and $C\delta$-deficient.

[0075] For instance, Ig molecules of class M are known to play an important role in the activation of macrophages and the complement pathway. Due to the close proximity of its binding sites, IgM has a high avidity for pathogens, including viruses. However, IgM is also known to be difficult for use in rapid immunoassay techniques whereas Ig of class G can be readily used in these techniques. For such uses, it would be useful to select for the preferred antibody class, ie IgG or IgM.

[0076] The expression of all or part of a heterologous heavy chain C_γ locus devoid of C_H1 will produce optionally some or all IgG isotypes, dependent on the IgG1, IgG2, IgG3 and IgG4 isotypes present in the heterologous IgG locus. Alternatively the heavy chains may comprise $C\varepsilon$ genes. The resulting IgE molecule might also be used in therapy.

[0077] Alternatively, selected mixtures of antibodies may be obtained. For example, IgA and IgM may be obtained when the heavy chain constant region comprises a $C\alpha$ and a $C\mu$ gene.

[0078] The heavy chain constant region may be of human origin, in particular when the heavy chain antibody is to be used for therapeutic applications in humans. Where the heavy chain antibodies are to be used for diagnostic or veterinary purposes, the heavy chain constant region is preferably derived from the target organism, vertebrate or mammal in or on which diagnosis or veterinary therapy is to be performed.

[0079] When expressed, the heavy chain constant region lacks a functional C_H1 domain. The C_H1 exon and, optionally, $C\mu$ and $C\delta$ constant regions, may be mutated, deleted or substituted. Preferably, the C_H1 exon is deleted. The presence, for example, of IgM with a functional C_H1 domain inhibits B-cell maturation and consequently limits the productive expression of heavy chain only IgG (devoid of C_H1) within the same locus, as B-cell maturation is inhibited.

[0080] A 'heavy chain constant region exon' ('C_H exon') as herein defined includes the sequences of naturally occurring vertebrate, but especially mammalian, C_H exons. This varies in a class specific manner. For example, IgG and IgA are naturally devoid of a C_H4 domain. The term 'C_H exon' also includes within its scope derivatives, homologues and fragments thereof in so far as the C_H exon is able to form a functional heavy chain-only antibody as herein defined when it is a component of a heavy chain constant region.

[0081] Optionally, when present, the C_H4 or other functional domains maybe engineered or deleted within the transgene provided such a process does not inhibit the intracellular secretory process, B-cell maturation or the binding activity of the resultant antibody polypeptide.

Rats

[0082] The transgenic mammal used in the methods of the invention is a rat.

[0083] Preferably transgenic rats are generated using established oocyte injection technology and, where established, ES cell technology or cloning.

[0084] Advantageously, immunoglobulin heavy and optionally light chain loci endogenous to the rat are deleted or silenced when a heavy chain-only antibody is expressed according to the methods of the invention.

[0085] This approach of generating heavy chain-only antibodies as described above maybe of particular use in the generation of antibodies 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.

[0086] The transgenic rat may be engineered to have a reduced capacity to produce antibodies that include light chains.

[0087] Antibody-producing cells may be derived from transgenic rats according to the present invention and used, for example, in the preparation of hybridomas for the production of heavy chain-only antibodies as herein defined. In addition or alternatively, nucleic acid sequences may be isolated from transgenic rats according to the present invention and

used to produce V_H domain heavy chain-only chain antibodies or bi-specific/bi-functional complexes thereof, using recombinant DNA techniques which are familiar to those skilled in the art.

[0088] Alternatively or in addition, antigen-specific heavy chain-only antibodies may be generated by immunisation of a transgenic rat according to the present invention.

[0089] Thus in a further aspect, the present invention provides a method for the production of heavy chain-only antibodies by immunising a transgenic rat according to the present invention with an antigen.

Heavy chain-only antibodies and fragments thereof

[0090] The antigen-specific, heavy chain-only binding domain, ie a V_H binding domain, is expressed by the V_H locus as a result of recombination between single V, D and J gene segments followed subsequently by somatic mutation. According to this aspect of the invention V_H loci can be cloned from, eg, mRNA isolated from an antibody-producing cell of an immunised transgenic rat as described above. Cloned sequences can then be displayed using a phage (Ward et al., (1989) Nature, 341, 544-546) or similar display libraries, for example using yeast-based systems (Boder and Wittrup, (1997) Nat. Biotechnol., 15, 553-7) and antigen-specific V_H binding domains identified. Antigen-specific heavy chain binding domains can then be manufactured either alone or as fusion proteins in scalable bacterial, yeast or alternative expression systems. Sequences encoding V_H binding domains can also be cloned from characterised hybridomas derived by classical procedures from immunised transgenic mice. These can then be used for the production of V_H binding domains and derivatives thereof including the engineering of defined antibody classes (eg IgE or IgA) and variants thereof with differing effector functions.

[0091] Accordingly, the invention also provides a method of producing a V_H binding domain comprising the steps of:

a) isolating a cell or tissue expressing an antigen-specific heavy chain-only antibody of interest (preferably a soluble, antigen-specific heavy chain-only antibody of interest);

b) cloning the sequence encoding the V_H binding domain from mRNA derived from the isolated cell or tissue;

c) displaying the encoded protein using a phage or similar library;

d) identifying antigen-specific V_H binding domains, and

e) expressing the V_H binding domains alone or as a fusion protein in bacterial, yeast, mammalian or alternative expression systems.

[0092] Alternatively, V_H domain-containing fragments can be generated from heavy chain-only antibodies of the invention using enzymic or chemical cleavage technology and subsequent separation of the V_H domain-containing fragment from the other cleavage products.

[0093] Where the V_H binding domain is isolated from a characterised hybridoma, the cloned V_H binding domain sequence derived from mRNA can be directly cloned into an expression vector without recourse to additional selection steps using phage and other display systems.

[0094] Production systems for heavy chain only-antibody incorporating effector regions include mammalian cells in culture (eg CHO cells), plants (eg maize), transgenic goats, rabbits, cattle, sheep, chickens and insect larvae suited to mass rearing technology. Other production systems, including virus infection (eg 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. Where there is a requirement for heavy chain-only IgA or IgM assembly, the co-expression of a "J chain" is beneficial. Suitable methods for the production V_H binding domains alone are known in the art. For example camelid V_H binding domains have been produced in bacterial systems and camelid heavy chain-only homodimers have been produced in hybridomas and transfected mammalian cells (see Reichmann and Muyldermans, (1999) J. Immunol. Methods, 231, 25-38).

[0095] Methods are also well established for the expression of engineered human V_H binding domains derived using phage display technology (Tanha et al., (2001) J. Biol. Chem., 276, 24774-24780 and references therein).

[0096] Insect larvae from transgenic fly lines have been shown to produce functional heavy chain-only antibody fragments in haemolymph with characteristics indistinguishable from the same antibody produced by mammalian cells (PCT/GB2003/0003319).

[0097] A heavy chain-only antibody produced by the method of the invention or a fragment thereof as herein described may be used as an intracellular binding reagent, or an abzyme.

[0098] An antigen-specific single chain antibody or VH binding domain produced according to the method of the invention may be used as an enzyme inhibitor or receptor blocker.

[0099] A V_H domain fused to an effector molecule may be used as a therapeutic, imaging agent, diagnostic, abzyme or reagent.

Brief Description of the Drawings

[0100]

Figure 1: shows the strategy for the generation of transgenic mice expressing an IgG locus and the functional generation of heavy chain-only antibodies and VH domains as a result of antigen challenge.

Figure 2: shows the strategy for the generation of transgenic mice expressing an IgM locus and the functional generation of heavy chain-only antibodies and VH domains as a result of antigen challenge.

Figure 3: shows the strategy for the generation of transgenic mice expressing an IgA locus and the functional generation of heavy chain-only antibodies and VH domains as a result of antigen challenge.

Figure 4: Sequence alignment of the PCR products obtained from bone marrow cDNA using $V_{HH}1$ and $V_{HH}2$ primers in combination with human $C\gamma 2$ primer from mice containing a locus with constant regions that have a camelid splice mutation to remove CH1. The results show that CH1 is not removed.

Figures 5-8: Structure of VH/camelid VH (VHH) constructs. 1-n stands for any number of VH genes, or D or J segments. The normal complement of the human locus is 51 V genes, 25 functional D segments (plus 2 non functional ones) and 6 J segments. In case of a $C\mu$ (for IgM) or $C\varepsilon$ (for IgE) region there is no H region and there is an additional CH4 exon between CH3 and M1. The VH genes(s) have been mutated to provide solubility as described in the public domain
The VH genes, D and J segments and C exons are preferably human, but could be from any other species including camelids. In the latter case the camelid VH (VHH) genes would not be mutated as they are naturally soluble.

Figure 9: Mouse immunization schedule and antibody assay for the generation of heavy chain-only IgG against *E.coli* HSP70.

Figure 10: Flow cytometric analysis and immunohistochemistry results for spleen cells derived from transgenic mice.

Figure 11: Results of ELISA analysis of DKTP immunized transgenic mice and sequence analysis of resulting antibody library.

Figure 12: Examples of somatic mutations and VDJ rearrangement seen in immunized transgenic mice.

Figure 13: Results of immunostaining assay on Tet-on cell line transfected with response plasmid containing A5 antibody.

Figure 14: Results of Western bolt analysis of sera of transgenic mouse lines.

Figure 15: Size fractionation of human IgM mixed with human single chain IgM produced by the IgM plus IgG locus mice.

Figure 16: Results of ELISA analysis of single chain IgM and IgG antibodies raised against human TNFα.

General Techniques

[0101] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e. g. in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed., John Wiley & Sons, Inc.) and chemical methods. In addition Harlow & Lane, A Laboratory Manual, Cold Spring Harbor, N. Y, is referred to for standard Immunological Techniques.

[0102] Any suitable recombinant DNA technique may be used in the method of the present invention. Typical expression

vectors, such as plasmids, are constructed comprising DNA sequences coding for each of the chains of the polypeptide complex or antibody. Any suitable established techniques for enzymic and chemical fragmentation of immunoglobulins and separation of resultant fragments may be used.

[0103] Suitable expression vectors which may be used for cloning in bacterial systems include plasmids, such as Col E1, pcR1, pBR322, pACYC 184 and RP4, phage DNA or derivatives of any of these.

[0104] For use in cloning in yeast systems, suitable expression vectors include plasmids based on a 2 micron origin.

[0105] Any plasmid containing an appropriate mammalian gene promoter sequence may be used in cloning in mammalian systems. Insect or bacculoviral promoter sequences may be used for insect cell gene expression. Such vectors include plasmids derived from, for instance, pBR322, bovine papilloma virus, retroviruses, DNA viruses and vaccinia viruses.

[0106] Suitable host cells which may be used for expression of the polypeptide complex or antibody include bacteria, yeasts and eukaryotic cells, such as insect or mammalian cell lines, transgenic plants, insects, mammalian and other invertebrate or vertebrate expression systems.

Example 1

[0107] In preliminary experiments, transgenic mice were prepared to express a heavy chain locus wherein two llama V_{HH} exons were linked to the human heavy chain diversity (D) and joining (J) segments, followed by the Cμ, Cδ, Cγ2, Cγ3 human constant region genes and human heavy chain immunoglobulin 3' LCR. The human Cγ2 and Cγ3 genes contained a G to A splice mutation. The presence of the Frt site enabled the generation of a single copy transgenic mouse from a multi-copy transgene array by Flp mediated recombination. However, sequences from the transgenic locus with a G to A splice mutation, showed aberrant splicing but incomplete CH1 removal (Figure 4).

Constructs

[0108] To overcome this problem, a genomic cosmid library was screened for clones containing the VH genes using standard methods. One (or more) different germline VHs were randomly chosen based on their sequence (five genera classes in the case of human VH's). Hydrophilic amino acid codons were introduced at positions 42, 49, 50 and 52 according to IMGT numbering (Lefranc *et al.* (1999)). The VH genes were combined into a BAC vector by standard procedures such as direct cloning using custom made linkers or homologous recombination.

[0109] Two clones were selected from the human genomic Pac library RPCI-11 (BACPAC Recource Center, USA): clone 1065 N8 containing human heavy chain D and J segments, Cμ (IgM) and Cδ (IgD) and clone 1115 N15 containing the Cγ3 (IgG3) genes. Bac clone 11771 from a different human genomic library (Incyte Genomics, CA, USA) was used as a source of Cγ2 (IgG2) gene and the immunoglobulin heavy chain LCR (Mills et al. (1997) J. Exp Med., 15;186(6):845-58).

[0110] Using standard techniques, the Cγ3 and Cγ2 genes were subcloned separately into pFastBac vector (Invitrogen). Similarly any of the other Ig constant regions can be cloned from these BACs (IgA, IgE). A complete deletion of CH1 exon was achieved by homologous recombination (Imam *et al.* (2001)) using sequences that flank the CH1 exon of each constant region. An frt site could optionally be introduced in front of the Cμ switch region to allow the generation of single copy loci from multicopy loci by treatment with flp recombinase in vivo by standard means e.g. by breeding to rosa-flp mice (Figure 5).

[0111] The separate VH genes, D and J segments and C and LCR exons were cloned into one BAC either by conventional restriction digestion and ligations or by homologous recombination (or a mixture of both) or any other cloning technique.

[0112] Further constructs could then be created.

IgM-only locus

[0113] In order to obtain the IgM construct (Figure 6), one or more VHs genes, followed by human D and J heavy chain segments and Cμ, were cloned into a BAC. For the methodology see above. In this case only the Cμ region was cloned into the final BAC.

IgMplus IgG locus, (Cδ is optional)

[0114] In order to obtain the IgM plus IgG construct (Figure 7), one or more VHs genes, followed by human D and J heavy chain segments, Cμ (without CH1 but with CH4 exon), (optional Cδ) and the modified human Cγ2 and Cγ3 genes and 3' LCR were cloned into a BAC. In order to generate an IgG only locus loxP sites were introduced during the standard cloning steps (described above) and the BAC is grown in 294 Cre E.coli strain (Buscholz *et al.*) and cre mediated

recombination yields bacteria producing an IgG only locus. For further construction details see above.

IgMplus IgG locus (Cδ is optional)

[0115] In order to obtain the IgM plus IgG construct (Figure 8), one or more VHs genes, followed by human D and J heavy chain segments, Cμ (with CH1 and CH4), (optional Cδ) and the modified human Cγ2 and Cγ3 genes and 3' LCR were cloned into a BAC. In order to generate an IgG only locus loxP sites were introduced during the standard cloning steps (described above) and the BAC was grown in 294 Cre E.coli strain (Buscholz *et al.*) and cre mediated recombination yielded bacteria producing an IgG only locus.

Transgenic mice, breeding and genotyping

[0116] The final BAC was introduced into transgenic mice by standard microinjection of fertilized eggs or via embryonic stem cell transfection technology.

[0117] Transgenic loci were checked for integrity and number of copies by Southern blot analysis of tail DNA (Southern 1975) using 5' and 3'end locus probes. Founders were bred as lines in the μMT-/- background. Genotyping was done by standard PCR analysis using primers for each of the different regions of the locus. Sequence analysis of the RT-PCR products derived from BM cDNA of transgenic mice where the entire CH1 exon from both the Cγ2 and the Cγ3 was been deleted (one with (HLL lines) and one without the Cμ and Cδ genes, showed that the transgenic loci are not only capable of VDJ recombination, but that the IgG transcripts resemble those found in llama and camel HCAbs.

Immunohistochemistry

[0118] Spleens were embedded in OCT compound. Frozen 5μm cryostat sections were fixed in acetone and single or double labeled as previously described (Leenen et al. 1998). Monoclonal antibodies anti B220/RA3-6B2, anti-CD11c/N418 (Steinman et al., 1997), were applied as hybridoma culture supernatants. Peroxidase coupled goat anti-human IgG and anti-human IgM were from Sigma. Second- step reagents were peroxidase labeled goat anti -rat Ig (DAKO, Glostrup, Denmark) or anti-hamster Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-rat Ig alkaline phosphatase (Southern Biotechnology, Birmingam, AL, USA).

[0119] Figure 10 shows the immunohistochemical analysis of 5μm frozen sections of spleens from $\mu MT^{-/-}$, WT and HLL and HLL-MD transgenic mice in the $\mu MT^{-/-}$ background. Sections were stained with anti B220 (blue) for B cells and anti-CD11c/N418 (brown) for dendritic cells. Arrows indicate the location of small clusters of B cells.

Flow Cytometric Analyses

[0120] Single cell suspensions were prepared from lymphoid organs in PBS, as described previously (Slieker et al. 1993). Approximately $1x10^6$ cells were incubated with antibodies in PBS/ 0.5% bovine serum albumin (BSA) in 96 well plates for 30 min at 4°C. Cells were washed twice in PBS/0.5% BSA. For each sample, $3x10^4$ events were scored using a FACScan analyzer (Becton Dickinson, Sunnyvale, CA). FACS data were analyzed using CellQuest version 1.0 computer software. Four-color analysis was performed on a Becton Dickinson FACS Calibur. The following mAbs were obtained from BD Pharmingen (San Diego, CA): FITC conjugated anti B220-RA3-6B2, PE conjugated anti CD19. FACS scan data of spleen cells, stained with anti-CD19 and anti-B220 are displayed in the bottom panel of Figure 10.

[0121] On the left of the Figure is a representation of Flp recombination *in vivo* by breeding HLL lines to a FlpeR transgenic line and supporting FACS scan data on spleen cells of the recombinant, showing B cell rescue as seen in the directly generated original HLL-MD lines. On the right is a representation of Cre recombination *in vivo* by breeding to Cag Cre transgenic line and FACS data on spleen cells of the single copy recombinant.

Immunization and hybridoma production (Figure 9)

[0122] Transgenic mice containing a heavy chain only antibody locus consisting of two llama VHH domains, human D and J regions and IgG2 and 3 constant regions (without a CH1 domain) were created.
8 week old mice were immunized with either E.Coli heat shock protein 70 (hsp70). 20 μg or 5μg of antigen with Specol adjuvant (IDDLO, Lelystadt, NL) was injected respectively s.c. on days 0, 14, 28, 42 and i.p. on day 50. Blood was taken on day 0, 14 and 45. After three boosts a low titer of antigen specific antibodies was detected in 1 out of 3 Hsp70 immunized HLL-MD1 mice (Figure 9).

[0123] A standard spleen cell fusion with a myeloma cell line was performed to generate a monoclonal antibody resulting in a monoclonal hybridoma cell line against the hsp70 protein. The anti-HSP 70 HCAb consists of the llama VHH segment closest to the D region (VHH 2) recombined to the human IgHD3-10 segment (acc.num. X13972) and

the human IgHJ4-02 segment (acc.num.X86355). Although not at high frequency, the VHHs has a few mutations that give rise to the amino acid alterations seen in Figure 4A when compared to the germ line configuration. The RT-PCR analysis also showed only one productive IgH transcript in the hybridoma, suggesting that there are no other transcripts made. The αHSP70 IgG2 antibody is secreted as heavy chain only dimer (Western blots under denaturing gel (dimer) and non denaturing gel (monomer) conditions Fig. 9). Spleen cells were fused with Sp2-O-Ag14 myeloma cells (gift from R. Haperen) on day 56 using a ClonalCellTM-HY kit (StemCell Technologies, UK) according to the manufacturer's instructions.

[0124] Transgenic mice containing a heavy chain only antibody locus consisting of two llama VHH domains, human D and J regions, a human IgM and IgG2 and 3 constant regions (all without a CH1 domain, Figure 7) were immunized with TNFα to obtain HC-IgM antibodies. One out of three mice showed positive sera in standard ELISA assays. A standard myeloma fusion yielded a positive IgM hybridoma (Figure 11). After gel filtration on Sepharose 6B under non-reduced conditions each fraction was of the column was loaded to a gel under reducing conditions and detected by αhuman IgM-HRP (Figure 15). Fractionation under non reducing conditions showed that the HC-IgM is secreted as a multimeric antibody with the same size as a human control IgM (after subtraction of the molecular weight of light chains and the CH1 domain that are absent from the HC-IgM). The gel fractionation of each column fraction under reducing conditions showed the expected monomer of (Fig. 15).

Serum Ig ELISA

[0125] Blood from 15-25 weeks old mice was collected in EDTA coated tubes, spun for 15' at room temperature (RT) and the supernatant diluted 1:5 in PBS. A 96 well plate was coated for 2h with 5mg/ml of a goat anti human IgG (YES Biotechnology) or a goat anti human IgM (Sigma), washed with PBS, blocked for 1h at RT with blocking solution (1.5% BSA/1.5% powder milk/0.1% tween 20/PBS) and washed three times with PBS. Dilution series of serum samples and standards (human IgG2 or human IgM (Sigma, Zwijndrecht, NL)) were loaded and incubated for 2-4h and the plates washed 6 times with PBS before addition of a secondary antibody (1:2000 diluted goat anti human IgG or goat anti human IgM coupled to HRP (Sigma, Zwijndrecht, NL)). All dilutions were done in a blocking solution. After 1-2h incubation at RT and washing in PBS, POD substrate (Roche) was added.

[0126] The ELISA for the detection of antigen specific soluble sdAbs from the IgG2 phage library is shown in Figure 11. Soluble sdAbs were used as primary antibodies on antigen-coated plates, followed by mouse α-myc antibody and HRP conjugated goat α-mouse antibody. POD was used as a substrate. The bottom panel shows fingerprinting of clones with restriction enzyme Hinf I, showing 5 different inserts coding for sdAb against *B.Pertusis.*

Antibody library construction and screening

[0127] Total RNA was isolated from spleens of DKTP immunized single copy IgG only mice (Figure 7 after cre treatment) using an Ultraspec RNA isolation system (Biotecx Laboratories Inc, Houston, Texas, USA). cDNA was made using oligo dT. DNA fragments encoding VHHDJ fragments were amplified by PCR using specific primers: vh1 back Sfi I primer (Dekker et al 2003) in combination with hIgG2hingrev primer (5'-AATCTGGGCAGCGGCCGCCTCGACACAACATTT-GCGCTC-3'). The amplified VHHDJs (~ 400 bp) were Sfi I / Not I digested, gel purified and cloned into Sfi I / NotI digested phagemid vector pHEN-1.

Transformation into TG1 electro-competent cells yielded in a human single domain antibody library. Two rounds of selection were performed using panning on vaccine antigens adsorbed onto plastic (immunotubes coated with undiluted vaccine). Restriction analysis and sequencing were standard.

RT-PCR of heavy chain-only locus

[0128] It was then investigated whether HLL-MD locus functions as a normal locus in producing a diverse antibody repertoire by sequencing the RT PCR products obtained using IgG2 and IgG3 specific primers on cDNA from Peyer's patches. Figure 12 shows some examples of somatic mutations of clones from non immunized mice (left panel) and immunized mice (right panel). The mice were IgG only loci, immunized E. Coli hsp70, Pertussis lysate, tetanus toxoid. In grey shade is the IgG2 hinge region starting with ERKCCV

[0129] Although, the RT-PCR analysis on Peyer's patches showed that both V_H are used, all the antibodies sequenced rearranged the VH2. The source of repertoire variability is the CDR3 region formed by the selection of D and J segments and by the V-D and D-J junctions. The use of human J segments is similar to that seen in human rearrangements, with the JH4 and JH6 segments being used most often.

[0130] This analysis showed that both VHs, different human D and all of the human J segments are used, to contribute to a diverse antibody repertoire. It also showed the presence of IgG3 switched B cells and the occurrence of somatic mutations by comparison of each rearranged gene with its germline counterpart i.e. the original V_H in the transgenic

construct (see Figure 12). Therefore, the human heavy chain-only IgG antigen receptor can provide the necessary signals for B cell maturation.

Immunostaining

[0131] Figure 13 shows immunostaining results of one of Tet- on cell line additionally transfected with the response plasmid containing A5 antibody (Dekker et al. 2003). The upper panel shows doxycicline induced production of A5 antibody (red) in cytoplasm and nuclear staining of the cells with DAPI (blue). Lower panel shows that cells expressing rtTA in nucleus are the ones producing the A5 upon induction (upper panel). Staining was done with one of the human HCAb against rtTA (green) with the sequence shown below. The FITC conjugated goat anti human IgG was used as a secondary step. A5 was detected as previously described by Dekker et al 2003. The rTTA antibody was an IgG3 with the following sequence:

241 AGACTCT

80 R L

301 CCTGTGCAGCCTCTGGAAGCATCTTCAGTATCAATGCCATGGGCTGGTACCGCCAGGCTC

100 S C A A S G S I F S I N A M G W Y R Q A

361 CAGGGAAGCAGCGCGAGTTGGTCGCAGCTATTACTAGTGGTGGTAGCACAAGGTATGCAG

120 P G K Q R E L V A A I T S G G S T R Y A

421 ACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAACACGGTGTATCTGC

140 D S V K G R F T I S R D N A K N T V Y L

481 AAATGAACAGCCTGAAACCTGAGGACACGGCCGTCTATTACTGTTTGATCTCTATGGTTC

160 Q M N S L K P E D T A V Y Y C L I S M V

541 GGGGAGCCCGTTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGAGCTCA

180 R G A R F D Y W G Q G T L V T V S S E L

601 AAACCCCACTT

200 K T P L

[0132] The IgG3 hinge starts at amino acid 198 ELKTPL. For comparison see the IgG2 hinge region in Figure 12.

Western blot analyses

[0133] Figure 14 shows Western blots of sera of different transgenic mouse lines containing the IgM plus IgG locus (Figure 5) after cre treatment (ie IgM deleted, only IgG left). Sera were purified by prot G and gel fractionated under reducing (Figure 14 right panel) and non reducing (Figure 14, left panel) conditions. The controls were the background KO mice and a normal human serum sample. Note the size difference between the two gels showing that the human heavy chain only IgG is a dimer.

[0134] The signal shown in Figure 14 was detected with an anti-human IgG antibody by standard procedures.

Size fractionation of human IgM produced by the IgM plus IgG locus mouse

[0135] The serum from the IgM plus IgG mice (Figure 8) was fractionated by gel filtration under non reducing conditions after mixing with a human serum sample as a control. Results are shown in Figure 15. Molecular weights of the complexes on the column decrease with each lane (representing each fraction) from left to right. The fractions (each lane) were analysed by gel electrophoresis under reducing conditions.

[0136] ELISA analysis was performed on a number of hybridomas made from mice containing the IgM plus IgG (Figure 8) locus immunized with human TNFα. Results are shown in Figure 16. The top two rows in Figure 16 were analysed with an anti-human IgG, the next two rows with an anti human IgM. The serum samples (arrows) show that the mouse has generated both IgG and IgM anti-TNFα antibodies. The single arrow shows a positive IgM hybridoma. The wells were coated with commercially available human TNFα. All procedures were standard.

SEQUENCE LISTING

[0137]

<110> ERASMUS UNIVERSITY MEDICAL CENTER ROTTERDAM

<110> CRAIG, Roger Kingdon

<120> BINDING MOLECULES

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1               5                  10                  15

Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val Ala Ala
            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Arg Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Leu Ile Ser
65                  70                  75                  80

Met Val Arg Gly Ala Arg Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
                85                  90                  95

                Thr Val Ser Ser Glu Leu Lys Thr Pro Leu
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ccatcggtct tccccctggc gccctgctcc aggagcacct ccgagagcac agcggccctg   180
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agcagcgtgg tgaccgtgcc ctccagcaac ttcggcaccc agacctacac ctgcaacgta   360
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cggccctggg ctgcctggtc aaggactact tccccgaacc ggtgacggtg tcgtggaact  180
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gcgtgcacac cttcccagct gtcctacagt cctcaggact ctactccctc agcagcgtgg  300
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tgctccagga gcacctccga gagcacagcg gccctgggct gcctggtcaa ggactacttc  180
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ttactactac tacggtatgg acgtctgggg ccaagggacc acggtcaccg tctcctcagc  120
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            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Asn Ala Lys
65                  70                  75                  80

Gly Pro Ile Thr His Val Arg Gly Val His Tyr Trp Gly Gln Gly Thr
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Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Asn Ala Lys
65                  70                  75                  80

Thr Pro Ile Thr His Ile Arg Gly Val His His Trp Gly Gln Gly Thr
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Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val Ala Ala
            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Asn Ala Arg
65                  70                  75                  80

Thr Pro Ile Thr Val Val Arg Gly Val His Tyr Trp Gly Gln Gly Thr
                85                  90                  95

Leu Val Thr Val Ser Ser Glu Arg Lys Cys Cys Val
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            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Lys Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Ser Ala Val Tyr Tyr Cys Asn Arg Thr
65                  70                  75                  80

Gly Pro Ile Thr His Val Arg Gly Val Asp Tyr Trp Gly Arg Gly Thr
                85                  90                  95

Leu Val Thr Val Ser Ser Glu Arg Lys Cys Cys Val
            100                 105
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Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val Ala Ala
            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn His Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Asn Ala Glu
65                  70                  75                  80

Ser Pro Ile Thr Lys Val Arg Gly Val Ser Tyr Trp Gly Gln Gly Thr
                85                  90                  95

Leu Val Thr Val Ser Ser Glu Arg Lys Cys Cys Val
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            20                  25                  30

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        35                  40                  45

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Gly Trp Phe Arg Gln Ala Glu Gly Lys Glu Arg Glu Gly Val Ser Cys
            20                  25                  30

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        35                  40                  45

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Gly Trp Phe Arg Gln Ala Glu Gly Lys Glu Arg Glu Gly Val Ser Cys
            20                  25                  30

Ile Ser Ser Ser Asp Gly Ser Thr Tyr Tyr Ala Asp Ser Val Lys Gly
        35                  40                  45

Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
    50                  55
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Gly Trp Phe Arg Gln Ala Glu Gly Lys Glu Arg Glu Gly Val Ser Cys
            20                  25                  30

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        35                  40                  45

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1               5                   10                  15

Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val Ala Ala
            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

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    50                  55
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        35                  40                  45

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1               5                   10                  15

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            20                  25                  30

Val Thr Ser Gly Gly Ser Thr Ser Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
    50                  55
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            20                  25                  30

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Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45
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```
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            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Asn Ala Glu
65                  70                  75                  80

Arg Ala Gly Asp Pro Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr
                85                  90                  95

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            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Gly Val Leu
65                  70                  75                  80

Trp Phe Gly Glu Leu Ser Asp Trp Phe Asp Tyr Trp Gly Gln Gly Thr
                85                  90                  95

Leu Val Thr Val Ser Ser Glu Arg Lys Cys Cys Val
            100                 105
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1               5                  10                  15

Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val Ala Ala
            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Asn Ala Asp
65                  70                  75                  80

Cys Trp Gly Ser Arg Trp Tyr Phe Asp His Tyr Trp Gly Arg Gly Thr
                85                  90                  95

Leu Val Thr Val Ser Ser Glu Arg Lys Cys Cys Val
            100                 105
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Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val Ala Ala
            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Asn Ala Asp
65                  70                  75                  80

Thr Ser Pro Pro Arg Tyr Phe Asp Trp Leu Pro Phe Asp Tyr Trp Gly
                85                  90                  95

Gln Gly Thr Leu Val Thr Val Ser Ser Glu Arg Lys Cys Cys Val
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1               5                  10                  15

Gly Trp Ser Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val Ala Ala
            20                  25                  30

Ile Thr Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg
        35                  40                  45

Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val His Leu Gln Met
    50                  55                  60

Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Asn Ala Gly
65                  70                  75                  80

Asn Thr Met Val Arg Gly Val Ile Ile Lys Tyr Arg Phe Asp Tyr Trp
                85                  90                  95

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Glu Arg Lys Cys Cys Val
            100                 105                 110
```

Claims

1. A method for the production of a V_H heavy chain-only antibody comprising:

   (a) immunising a transgenic rat expressing a heterologous V_H heavy chain locus with an antigen, wherein:

   (i) the V_H heavy chain locus comprises a variable region comprising at least one naturally-occurring V_H gene segment, at least one D gene segment, at least one J gene segment and at least one heavy chain constant region, and wherein the V, D and J gene segments are derived from a human;
   (ii) each constant region does not encode a C_H1 domain;
   (iii) a V_H gene segment, a D gene segment and a J gene segment are capable of recombining to form a VDJ coding sequence;
   (iv) the recombined V_H heavy chain locus, when expressed, is capable of forming a soluble, heavy chain-only antibody comprising a soluble, antigen-specific V_H binding domain and a constant effector region devoid of a C_H1 domain;

   (b) isolating a nucleic acid sequence encoding the V_H heavy chain-only antibody from antibody producing cells; and
   (c) producing the V_H heavy chain-only antibody using recombinant DNA techniques.

2. The method of claim 1, further comprising cloning a V_H locus encoding the V_H binding domain of the V_H heavy chain-only antibody and expressing the V_H binding domain in a bacterial, yeast, mammalian or alternative expression system.

3. The method of claim 1 or claim 2, wherein said transgenic rat has been engineered to have a reduced capacity to produce antibodies which include light chains.

4. The method of any one of claim 1 to 3, wherein immunoglobulin heavy chain loci endogenous to the rat are deleted or silenced.

5. The method of any one of the preceding claims, wherein the V_H heavy chain locus comprises more than one V_H gene segment, more than one D gene segment and more than one J gene segment.

Patentansprüche

1. Verfahren zur Herstellung eines Antikörpers mit lediglich Schwere-Kette-V_H, umfassend:

   (a) Immunisieren einer transgenen Ratte, die einen heterologen Schwere-Kette-V_H-Locus exprimiert, mit einem Antigen, wobei:

(i) der Schwere-Kette-V_H-Locus eine variable Region, die wenigstens ein natürlich vorkommendes V_H-Gensegment umfasst, wenigstens ein D-Gensegment, wenigstens ein J-Gensegment und wenigstens eine konstante Schwere-Kette-Region umfasst und wobei die V-, D- und J-Gensegmente von einem Menschen stammen;
(ii) die konstanten Regionen jeweils keine C_H1-Domäne codieren;
(iii) ein V_H-Gensegment, ein D-Gensegment und ein J-Gensegment unter Bildung einer VDJ-Codiersequenz rekombinieren können;
(iv) bei Expression des rekombinierten Schwere-Kette-V_H-Locus ein löslicher Antikörper mit lediglich schwerer Kette gebildet werden kann, der eine lösliche, antigenspezifische V_H-Bindungsdomäne und eine konstante Effektorregion ohne eine C_H1-Domäne umfasst;

(b) Isolieren einer Nukleinsäuresequenz, die den Antikörper mit lediglich Schwere-Kette-V_H codiert, aus Antikörper produzierenden Zellen; und
(c) Herstellen des Antikörpers mit lediglich Schwere-Kette-V_H unter Verwendung rekombinanter DNA-Techniken.

2. Verfahren nach Anspruch 1, ferner umfassend Klonieren eines die V_H-Bindungsdomäne des Antikörpers mit lediglich Schwere-Kette-V_H codierenden V_H-Locus und Exprimieren der V_H-Bindungsdomäne in einem bakteriellen, Hefe-, Säuger- oder alternativen Expressionssystem.

3. Verfahren nach Anspruch 1 oder Anspruch 2, wobei die transgene Ratte so konstruiert wurde, dass sie eine verminderte Kapazität zur Herstellung von Antikörpern, die leichte Ketten enthalten, besitzt.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei für die Ratte endogene Immunglobulin-schwere-Kette-Loci deletiert sind oder einem Silencing unterzogen wurden.

5. Verfahren nach einem der vorhergehenden Ansprüche, wobei der Schwere-Kette-V_H-Locus mehr als ein V_H-Gensegment, mehr als ein D-Gensegment und mehr als ein J-Gensegment umfasst.

Revendications

1. Procédé destiné à la production d'un anticorps à chaîne lourde uniquement avec V_H comprenant les étapes consistant à :

(a) immuniser un rat transgénique exprimant un locus hétérologue de V_H d'une chaîne lourde avec un antigène, dans lequel :

(i) le locus de V_H d'une chaîne lourde comprend une région variable, qui comprend au moins un segment de gène V_H existant naturellement, au moins un segment de gène D, au moins un segment de gène J et au moins une région constante de chaîne lourde, et dans lequel les segments géniques V, D et J sont dérivés d'un être humain ;
(ii) chaque région constante ne code pas pour un domaine C_H1 ;
(iii) un segment de gène V_H, un segment de gène D et segment de gène J sont capables de se recombiner pour former une séquence codante de VDJ ;
(iv) le locus recombiné de V_H d'une chaîne lourde, quand il est exprimé, est capable de former un anticorps soluble à chaîne lourde uniquement, qui comprend un domaine soluble de liaison V_H spécifique d'un antigène et une région effectrice constante dépourvue d'un domaine C_H1 ;

(b) isoler une séquence d'acides nucléiques codant pour l'anticorps à chaîne lourde uniquement avec V_H à partir de cellules productrices d'anticorps ; et
(c) produire l'anticorps à chaîne lourde uniquement avec V_H en utilisant des techniques d'ADN recombinant.

2. Procédé, selon la revendication 1, comprenant en outre le clonage d'un locus de V_H, qui code pour le domaine de liaison V_H de l'anticorps à chaîne lourde uniquement avec V_H, et l'expression du domaine de liaison V_H dans un système d'expression bactérien, de levure, mammalien ou alternatif.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel ledit rat transgénique a été génétiquement

manipulé pour avoir une capacité réduite de production d'anticorps qui comprennent des chaînes légères.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel les loci de chaînes lourdes des immunoglobulines endogènes au rat sont supprimés ou rendus silencieux.

5. Procédé selon l'une quelconque des revendications précédentes, dans lequel le locus de V_H d'une chaîne lourde comprend plus d'un segment de gène V_H, plus d'un segment de gène D et plus d'un segment de gène J.

FIG. 1

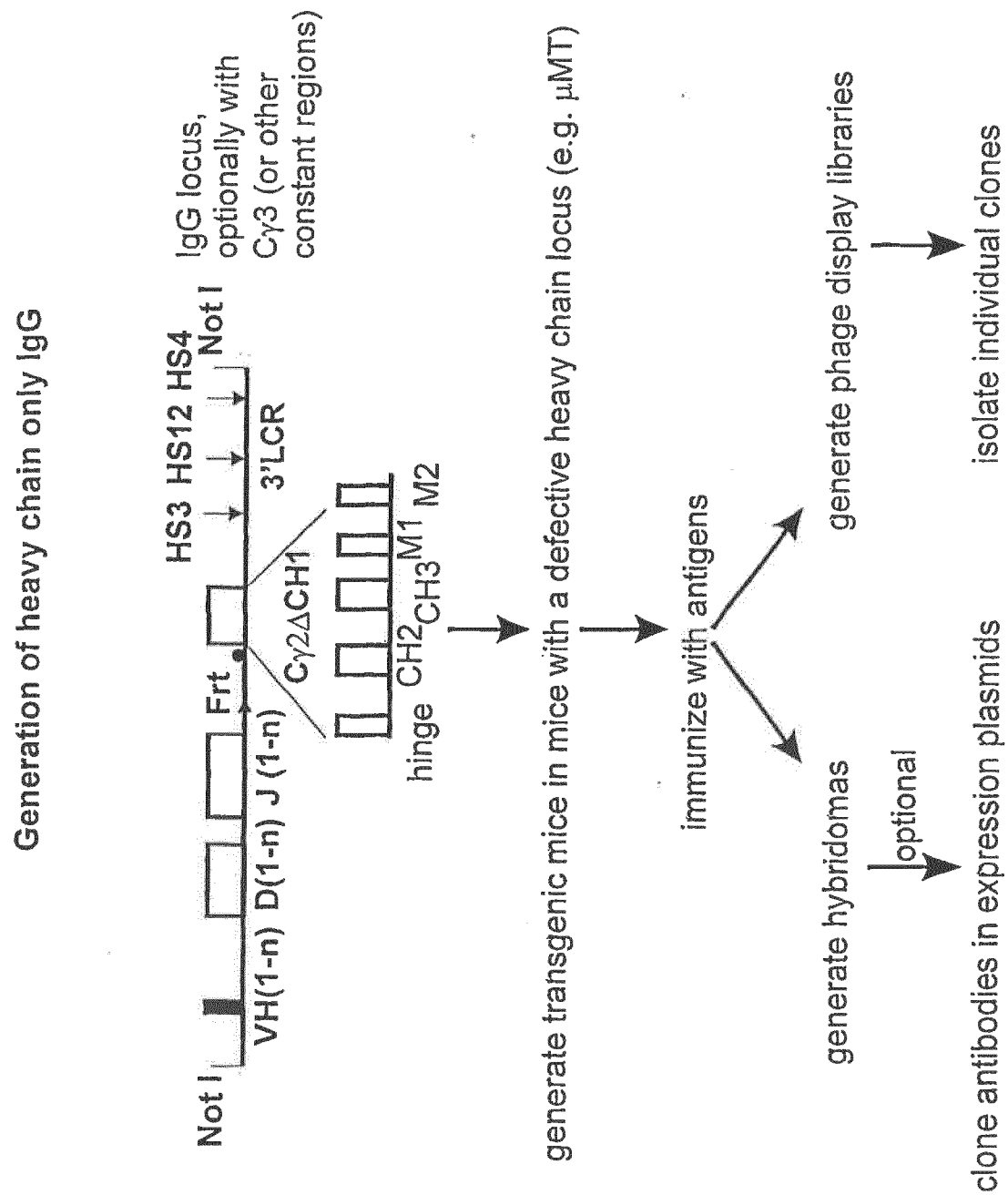

Generation of heavy chain only IgG
Not I
VH(1-n) D(1-n) J (1-n)
Frt
Cγ2ΔCH1
HS3 HS12 HS4
Not I
3'LCR
IgG locus, optionally with Cγ3 (or other constant regions)
hinge CH2CH3M1 M2
generate transgenic mice in mice with a defective heavy chain locus (e.g. μMT)
immunize with antigens
generate hybridomas
optional
clone antibodies in expression plasmids
generate phage display libraries
isolate individual clones

FIG. 2

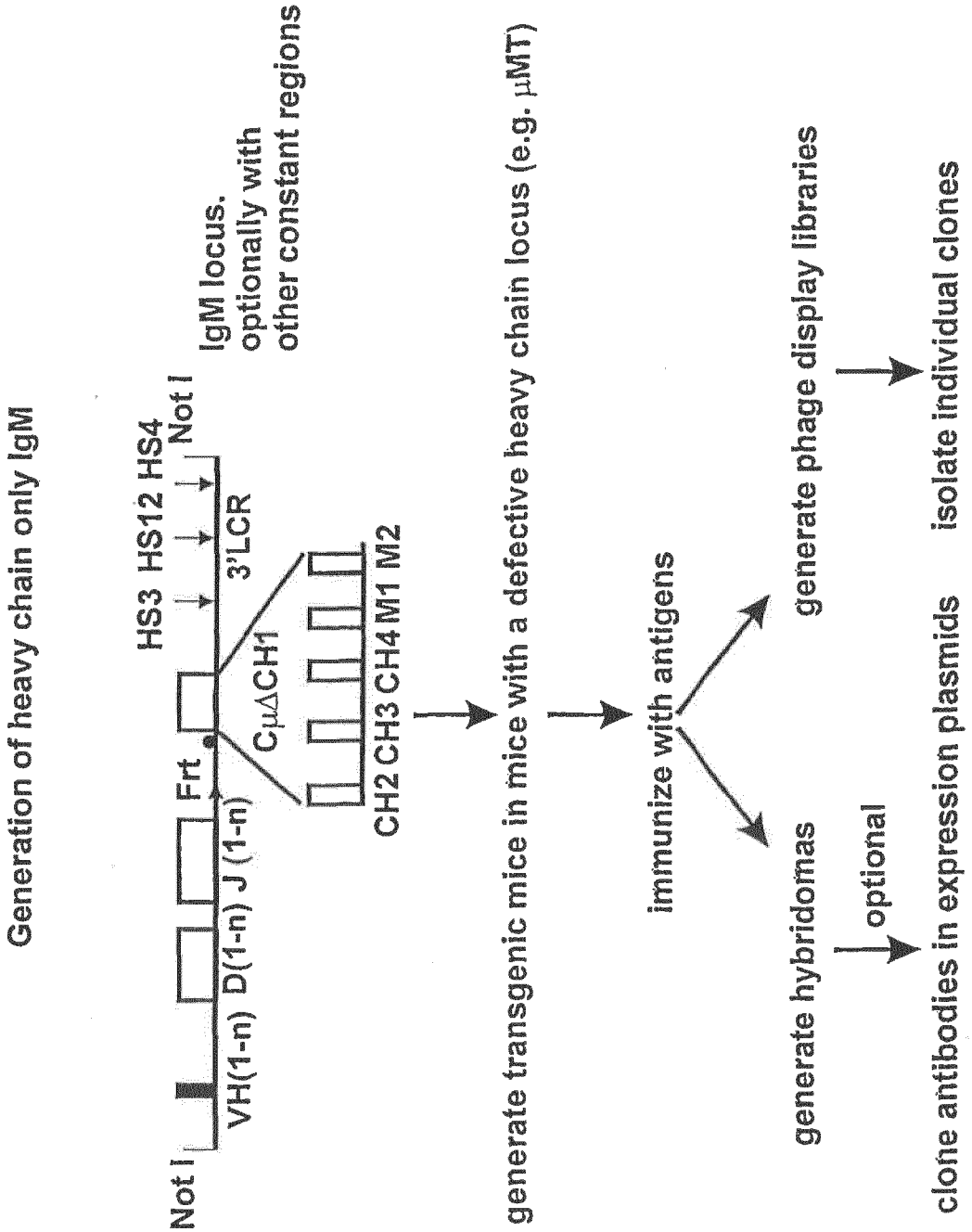

Generation of heavy chain only IgM
Not I
VH(1-n) D(1-n) J (1-n)
Frt
CμΔCH1
HS3 HS12 HS4
Not I
3'LCR
IgM locus,
optionally with
other constant regions
CH2 CH3 CH4 M1 M2
generate transgenic mice in mice with a defective heavy chain locus (e.g. μMT)
immunize with antigens
generate hybridomas
optional
clone antibodies in expression plasmids
generate phage display libraries
isolate individual clones

FIG. 3

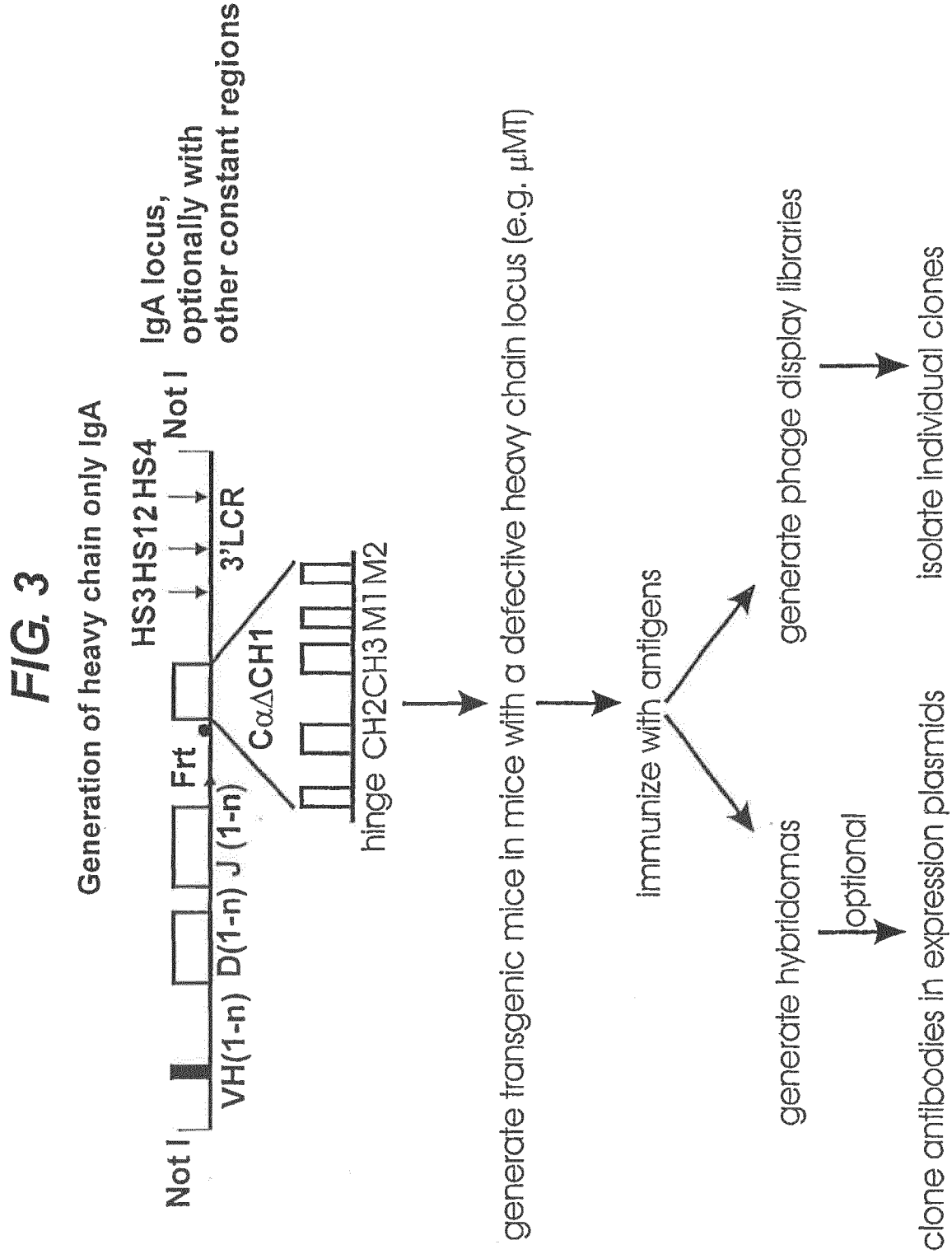

Generation of heavy chain only IgA
Not I
VH(1-n) D(1-n) J (1-n)
Frt
CαΔCH1
hinge CH2CH3 M1 M2
HS3 HS12 HS4
Not I
3'LCR
IgA locus,
optionally with
other constant regions
generate transgenic mice in mice with a defective heavy chain locus (e.g. μMT)
immunize with antigens
generate hybridomas
optional
clone antibodies in expression plasmids
generate phage display libraries
isolate individual clones

FIG. 4

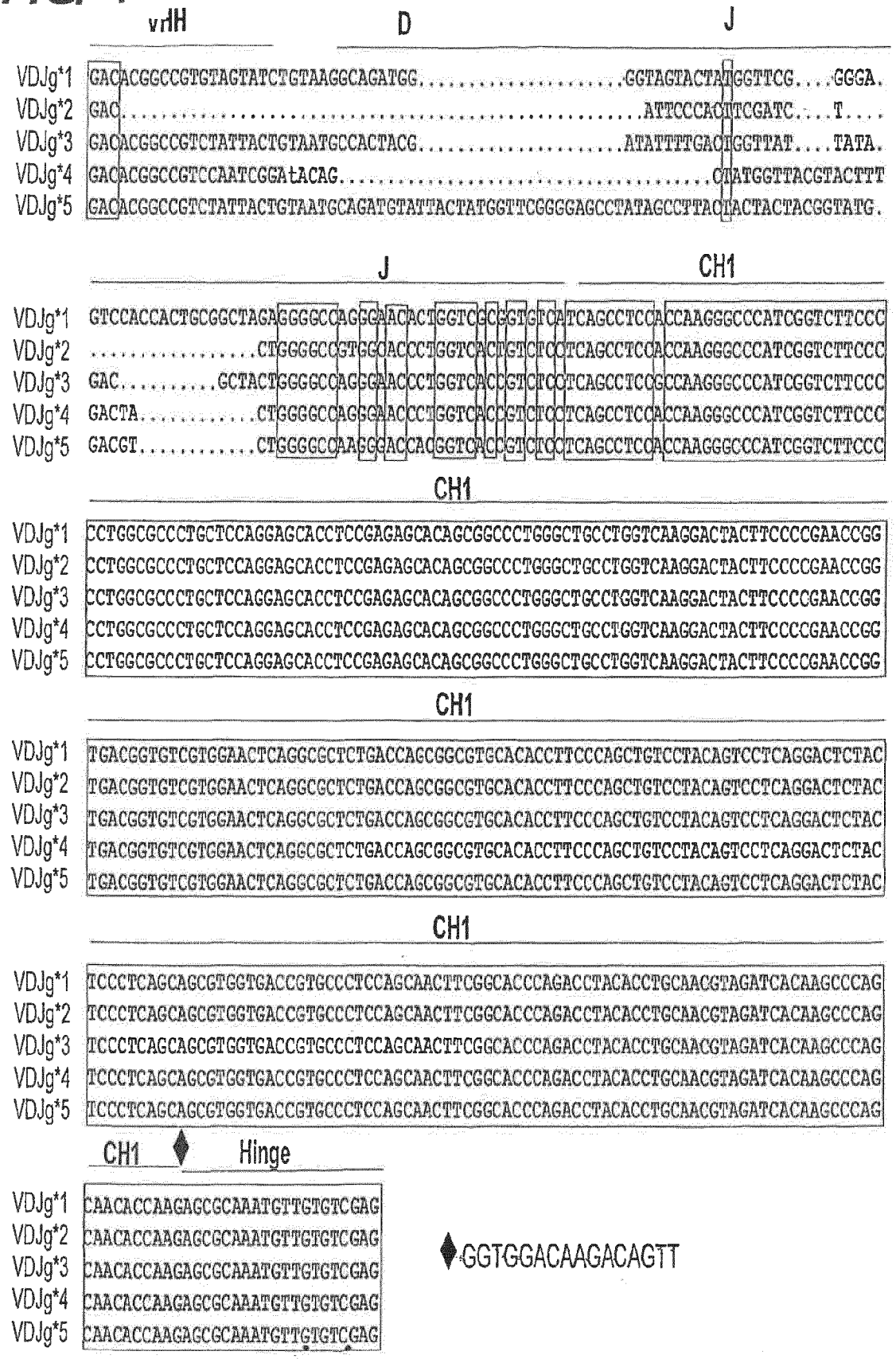

vHH
D
J
VDJg*1 GACACGGCCGTGTAGTATCTGTAAGGCAGATGG.....GGTAGTACTATGGTTCG....GGGA.
VDJg*2 GAC.....ATTCCCACTTCGATC....T....
VDJg*3 GACACGGCCGTCTATTACTGTAATGCCACTACG.....ATATTTTGACTGGTTAT....TATA.
VDJg*4 GACACGGCCGTCCAATCGGATACAG.....CTATGGTTACGTACTTT
VDJg*5 GACACGGCCGTCTATTACTGTAATGCAGATGTATTACTATGGTTCGGGGAGCCTATAGCCTTACTACTACTACGGTATG.
J
CH1
VDJg*1 GTCCACCACTGCGGCTAGAGGGGCCAGGCAACACTGGTCGCCGTGTCATCAGCCTCCACCAAGGGCCCATCGGTCTTCCC
VDJg*2 .....CTGGGGCCGTGGCACCCTGGTCACTGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCC
VDJg*3 GAC.....GCTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGCCTCCGCCAAGGGCCCATCGGTCTTCCC
VDJg*4 GACTA.....CTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCC
VDJg*5 GACGT.....CTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCC
CH1
VDJg*1 CCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
VDJg*2 CCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
VDJg*3 CCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
VDJg*4 CCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
VDJg*5 CCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
CH1
VDJg*1 TGACGGTGTCGTGGAACTCAGGCGCTCTGACCAGCGGCGTGCACACCTTCCCAGCTGTCCTACAGTCCTCAGGACTCTAC
VDJg*2 TGACGGTGTCGTGGAACTCAGGCGCTCTGACCAGCGGCGTGCACACCTTCCCAGCTGTCCTACAGTCCTCAGGACTCTAC
VDJg*3 TGACGGTGTCGTGGAACTCAGGCGCTCTGACCAGCGGCGTGCACACCTTCCCAGCTGTCCTACAGTCCTCAGGACTCTAC
VDJg*4 TGACGGTGTCGTGGAACTCAGGCGCTCTGACCAGCGGCGTGCACACCTTCCCAGCTGTCCTACAGTCCTCAGGACTCTAC
VDJg*5 TGACGGTGTCGTGGAACTCAGGCGCTCTGACCAGCGGCGTGCACACCTTCCCAGCTGTCCTACAGTCCTCAGGACTCTAC
CH1
VDJg*1 TCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAG
VDJg*2 TCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAG
VDJg*3 TCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAG
VDJg*4 TCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAG
VDJg*5 TCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAACGTAGATCACAAGCCCAG
CH1
Hinge
VDJg*1 CAACACCAAGAGCGCAAATGTTGTGTCGAG
VDJg*2 CAACACCAAGAGCGCAAATGTTGTGTCGAG
VDJg*3 CAACACCAAGAGCGCAAATGTTGTGTCGAG
VDJg*4 CAACACCAAGAGCGCAAATGTTGTGTCGAG
VDJg*5 CAACACCAAGAGCGCAAATGTTGTGTCGAG
GGTGGACAAGACAGTT

FIG. 5

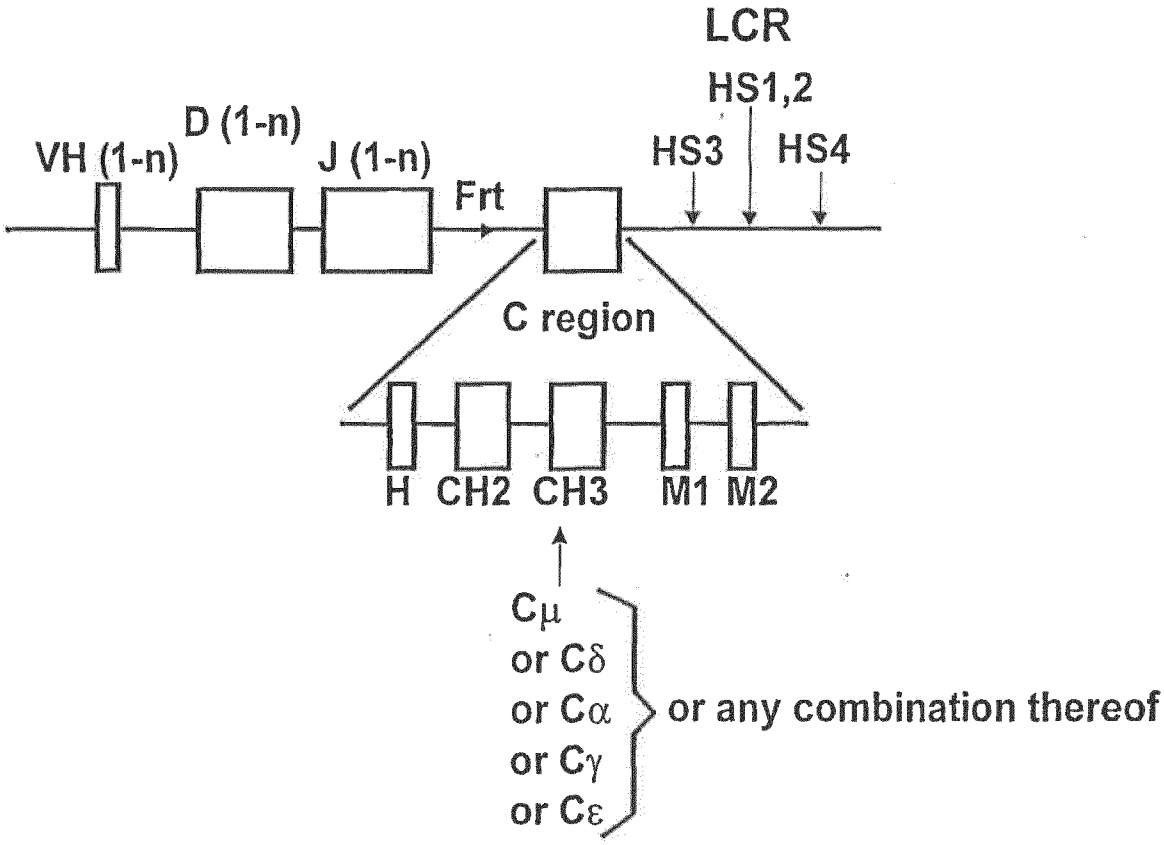

LCR
HS1,2
D (1-n)
VH (1-n)
J (1-n)
HS3
HS4
Frt
C region
H
CH2
CH3
M1
M2
Cμ
or Cδ
or Cα
or Cγ
or Cε
or any combination thereof

FIG. 6

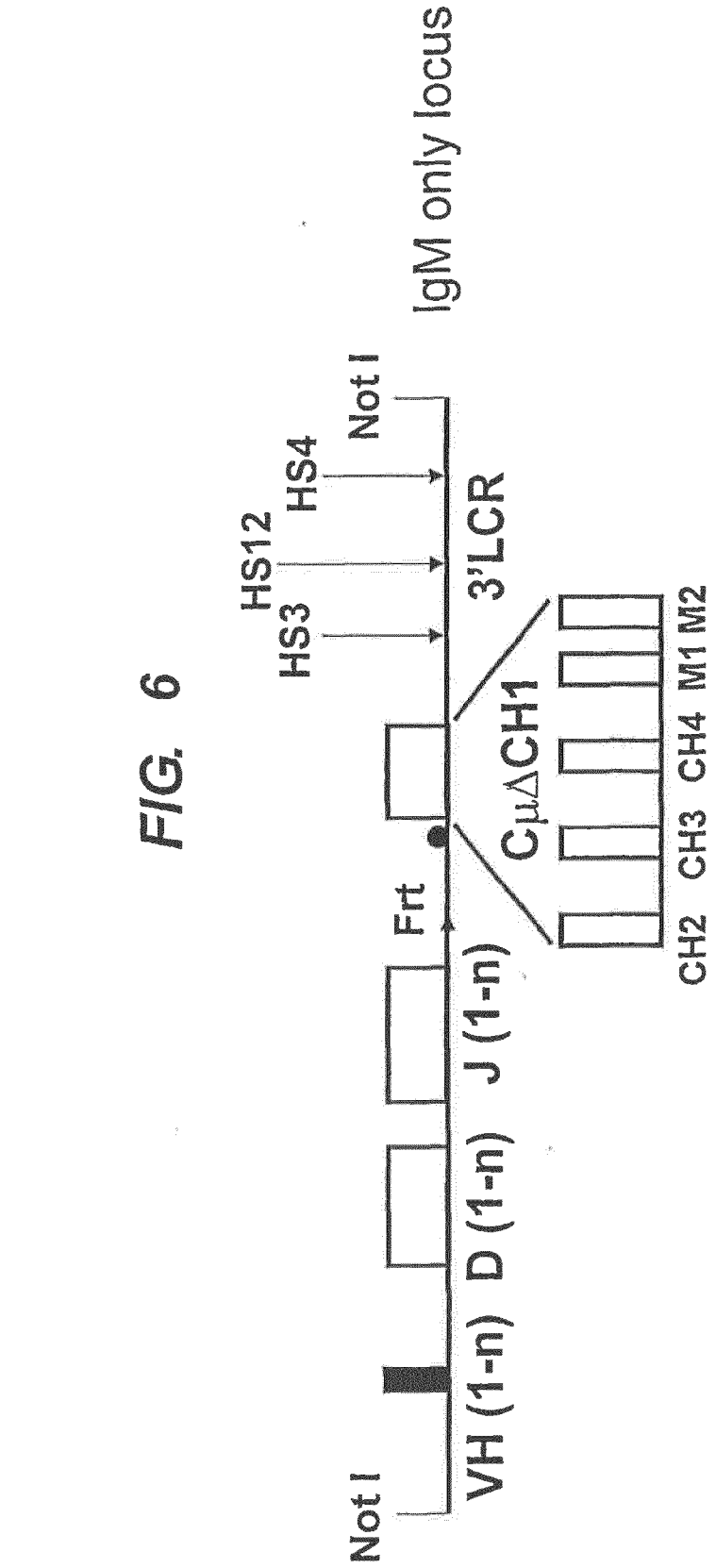
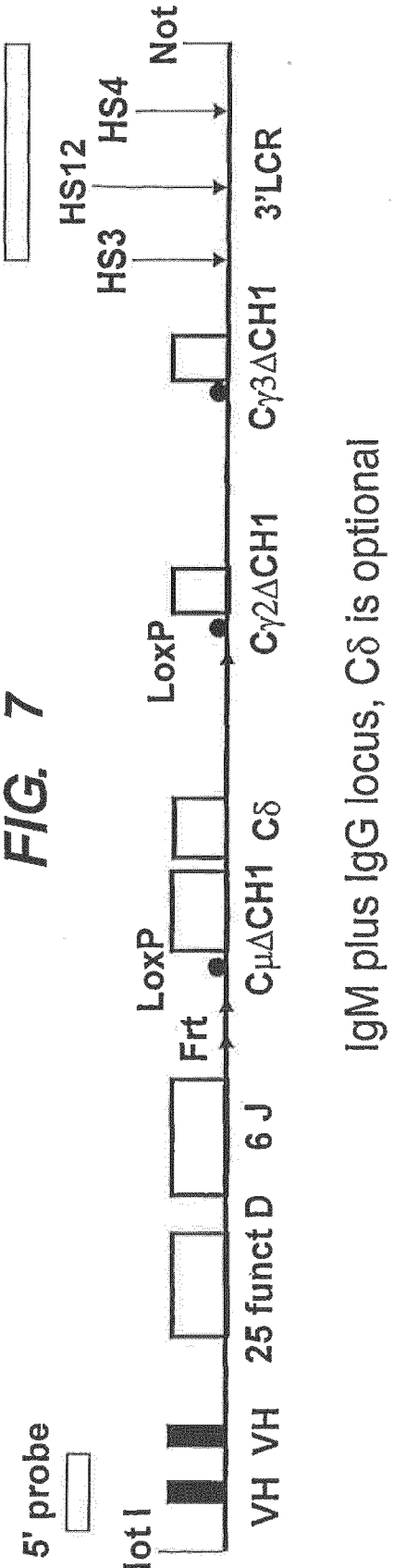

IgM only locus
Not I
HS4
HS12
HS3
3'LCR
CμΔCH1
Frt
J (1-n)
D (1-n)
VH (1-n)
Not I
CH2
CH3
CH4
M1
M2

FIG. 7



IgM plus IgG locus, Cδ is optional

FIG. 8

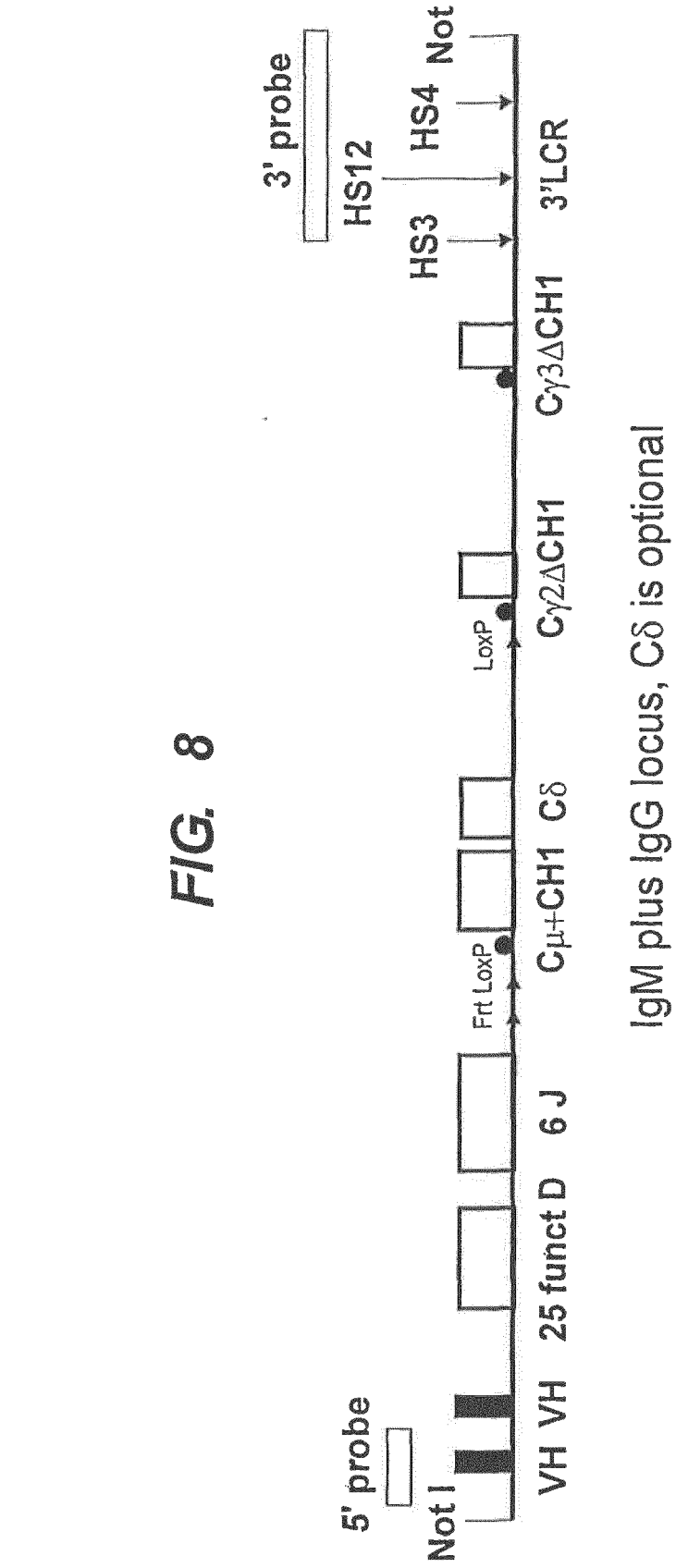
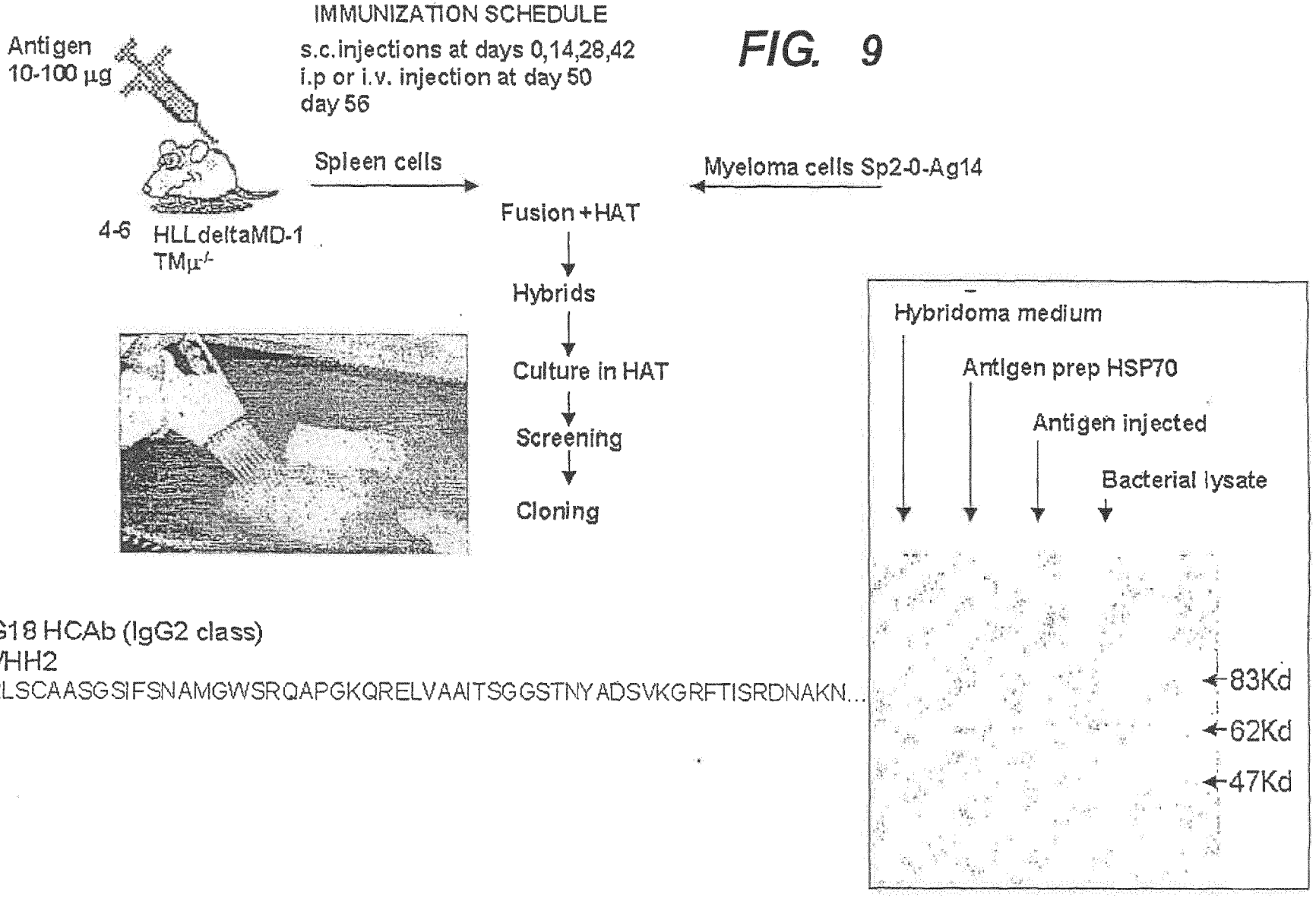

5' probe
Not I
VH VH 25 funct D 6 J
Frt LoxP
Cμ+CH1 Cδ
LoxP
Cγ2ΔCH1
Cγ3ΔCH1
HS3
HS12
HS4
3'LCR
Not
3' probe
IgM plus IgG locus, Cδ is optional

FIG. 9


IMMUNIZATION SCHEDULE
s.c.injections at days 0,14,28,42
i.p or i.v. injection at day 50
day 56
Antigen
10-100 µg
4-6
HLLdeltaMD-1
TMµ-/-
Spleen cells
Myeloma cells Sp2-0-Ag14
Fusion + HAT
Hybrids
Culture in HAT
Screening
Cloning
G18 HCAb (IgG2 class)
VHH2
RLSCAASGSIFSNAMGWSRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKN...
Hybridoma medium
Antigen prep HSP70
Antigen injected
Bacterial lysate
83Kd
62Kd
47Kd

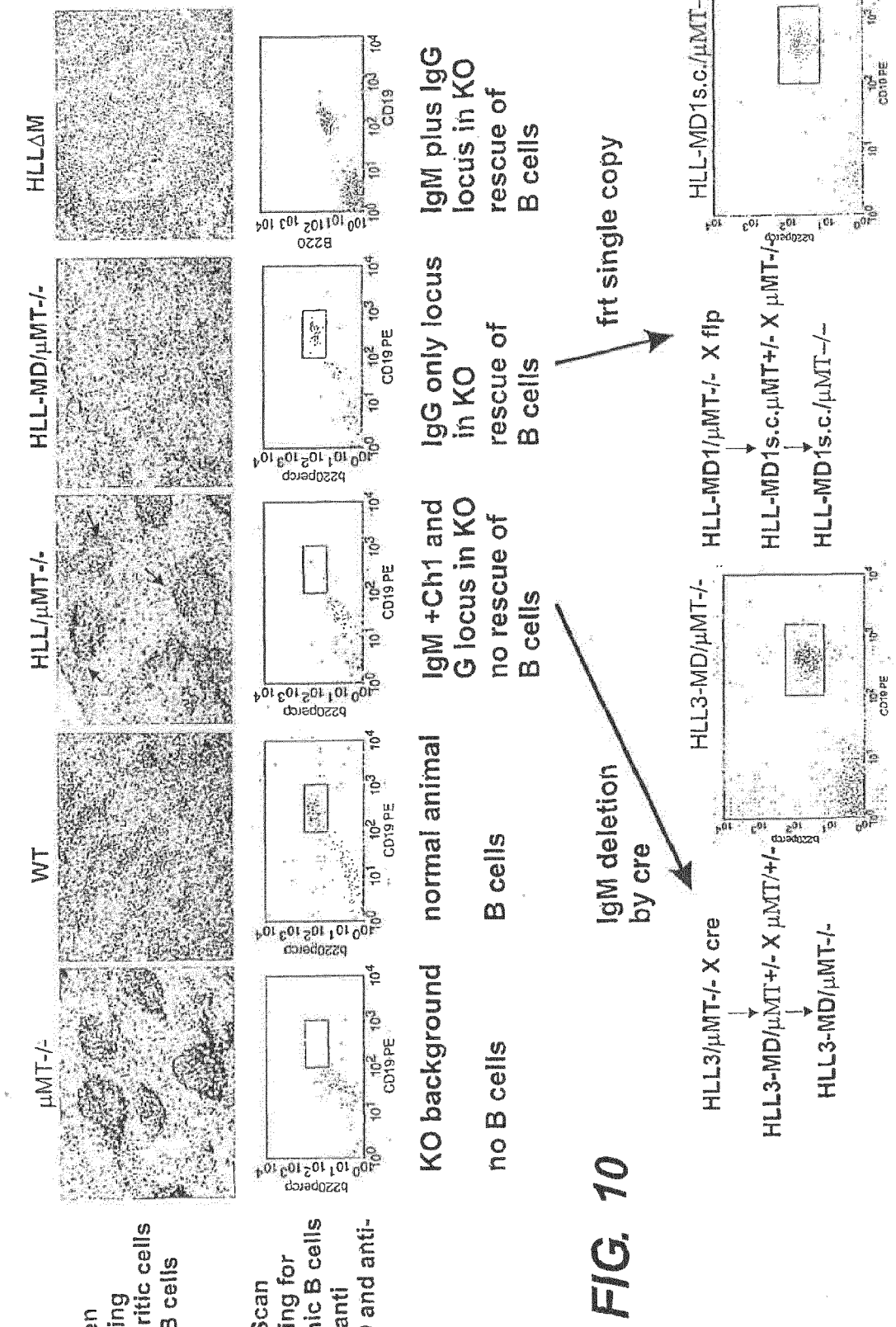

µMT-/-
WT
HLL/µMT-/-
HLL-MD/µMT-/-
HLLΔM
spleen staining dendritic cells and B cells
FACScan staining for splenic B cells with anti Cd19 and anti-B220
B220
CD19
CD19 PE
KO background
normal animal
IgM +Ch1 and G locus in KO
IgG only locus in KO
IgM plus IgG locus in KO
no B cells
B cells
no rescue of B cells
rescue of B cells
rescue of B cells
IgM deletion by cre
frt single copy
HLL3/µMT-/- X cre
HLL3-MD/µMT+/- X µMT/+/-
HLL3-MD/µMT-/-
HLL-MD1/µMT-/- X flp
HLL-MD1s.c.µMT+/- X µMT-/-
HLL-MD1s.c./µMT-/-
HLL-MD1s.c./µMT-/-

FIG. 10

FIG. 11

α-B.Pertussis

CDR3

1 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNAKGPITHVRG
2 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNAKTPITHIRG
3 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNARTPITVVRG
4 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDKAKNTVYLQMNSLKPEDSAVYYCNRTGPITHVRG
5 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNAESPITKVRG

CDR3 HINGE

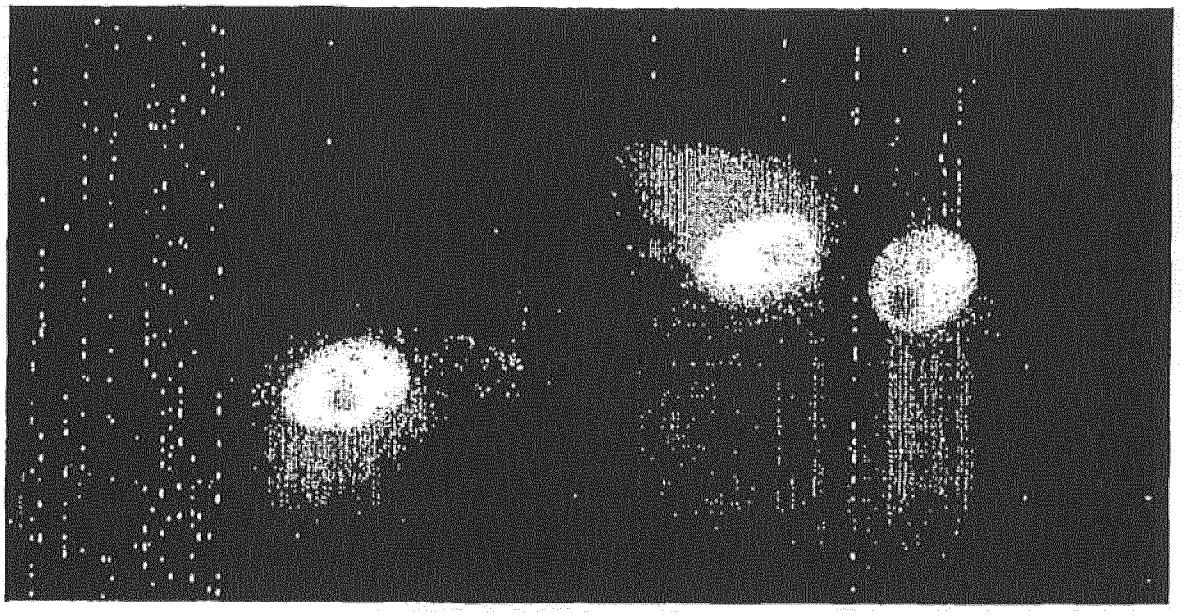
....VHYWGQGTLVTVSSERKCCV...
....VHHWGQGTLVTVSSERKCCV...
....VHYWGQGTLVTVSSERKCCV...
....VHYWGQGTLVTVSSERKCCV...
....VDYWGRGTLVTVSSERKCCV...
....VSYWGQGTLVTVSSERKCCV...

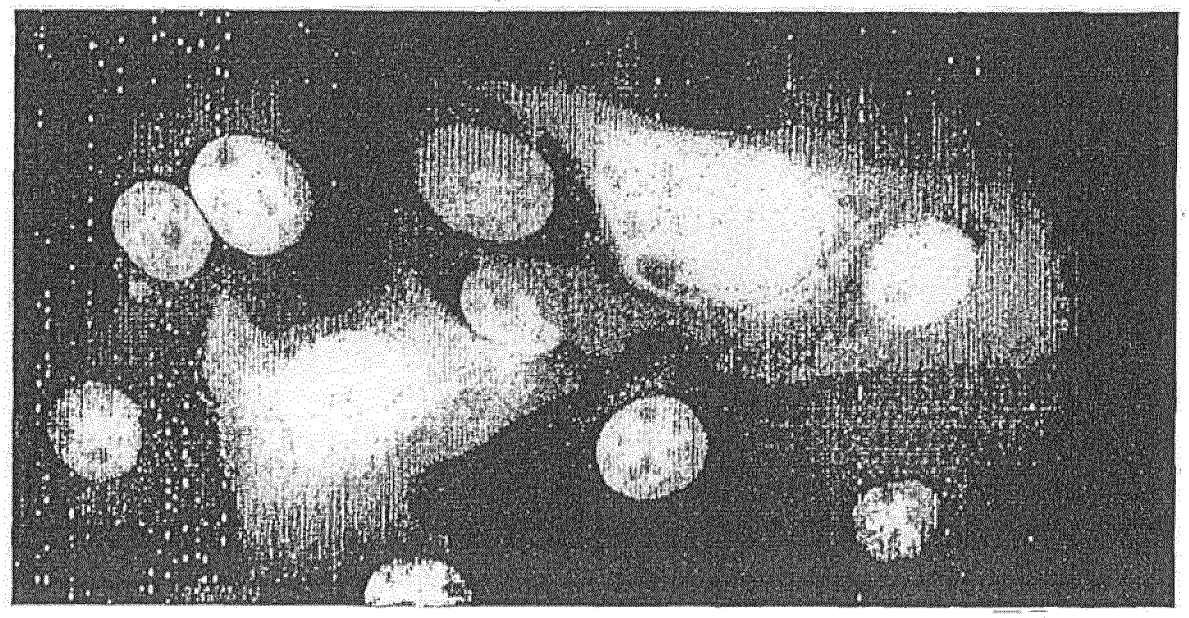
Sequence of clones indicated by an asterix in the fingerprint,
note the short CDR3 region


ne VHH1 RLSCAASGFTLDYYAIGWFRQAEGKEREGVSCISSSDGSTYYADSVKGRFTISRDNAKN
Clone 1 RLSCAASGFTLDYYAIGWFRQAEGKEREGVSCISSSDGSTYYADSVKGRFTISRDNAKN
Clone 2 RLSCAASGFTLDYYAIGWFRQAEGKEREGVSCISSSDGSTYYADSVKGRFTISRDNAKN
Clone 3 RLSCAASGFTLDYYAIGWFRQAEGKEREGVSCISSSDGSTYYGDSVKGRFTISRDKAKN
CDR1
CDR2
ne VHH2 RLSCAASGSIFSINAMGWYRQAPGKQRELVAA ITSGGSTNYADSVKGRFTISRDNAKN
Clone 1 RLSCAASGSIFSINAMGWYRQAPGKQRELVAA ITSGGSTKYADSVKGRFTISRDNAKN
Clone 2 RLSCAASGSIFSINVMGWYRQPPGKQRELVAGVTSGGSTSYADSVKGRFTISRDNAKN
Clone 3 RLSCAASGSIFSINAMGWYRQAPGKQRELVAP ITSGGSTNYADSVKGRFTISRDNAKN
CDR1
CDR2
CDR1
CDR2
VHH2 germline VHH2 RLSCAASGSIFSINAMGWYRQAPGKQRELVAA ITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCN.....
CDR3
α-B.Pertussis
1 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNAKGPITHVRG
2 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNAKTPITHIRG
3 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNARTPITVVRG
4 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDKAKNTVYLQMNSLKPEDSAVYYCNRTGPITHVRG
5 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNHADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNAESPITKVRG
α-Tetanus
1 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNAERAGDP.....
2 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCGVLWFGELSD..
3 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNADCWGSRW..
4 RLSCAASGSIFSINAMGWYRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNADTSPPRYFDW
α-hsp70 RLSCAASGSIFSSNAMGWSRQAPGKQRELVAAITSGGSTNYADSVKGRFTISRDNAKNTVHLQMNSLKPEDTAVYYCNAGNTMVRGVII
CDR3
HINGE
α-B.Pertussis
1....VHYWGQGTLVTVSSERKCCV...
2....VHHWGQGTLVTVSSERKCCV...
3....VHYWGQGTLVTVSSERKCCV...
4....VDYWGRGTLVTVSSERKCCV...
5....VSYWGQGTLVTVSSERKCCV...
α-Tetanus
1 .....FDYWGQGTLVTVSSERKCCV...
2 ..WFDYWGQGTLVTVSSERKCCV...
3 ...YFDHYWGRGTLVTVSSERKCCV...
4 .LPFDYWGQGTLVTVSSERKCCV...
α-hsp70 ....KYRFDYWGQGTLVTVSSERKCCV...

FIG. 12

FIG. 13





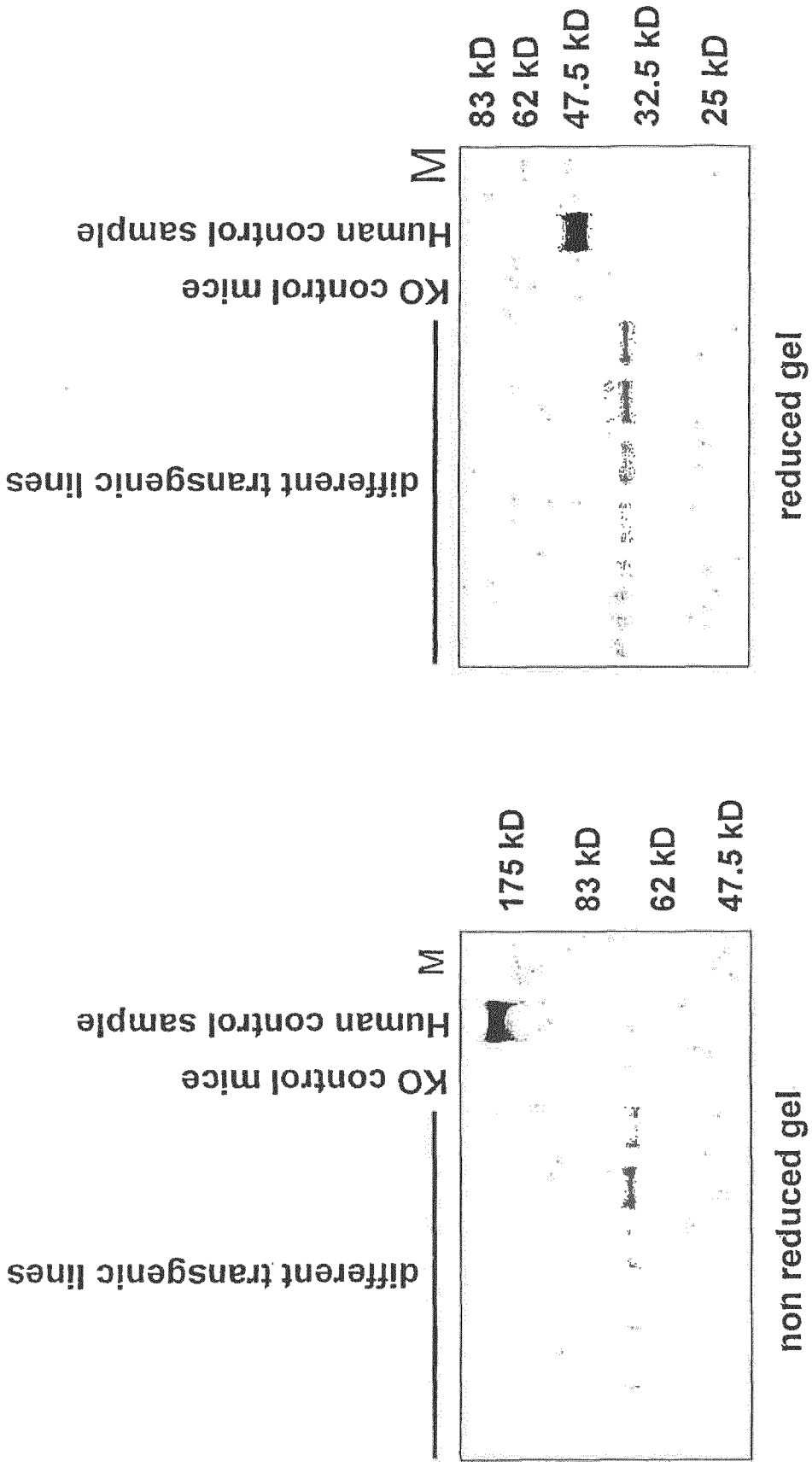

M
Human control sample
KO control mice
different transgenic lines
175 kD
83 kD
62 kD
47.5 kD
non reduced gel
M
Human control sample
KO control mice
different transgenic lines
83 kD
62 kD
47.5 kD
32.5 kD
25 kD
reduced gel

FIG. 14

FIG. 15

Size fractionation of human IgM mixed with human single chain IgM produced by the IgM plus IgG locus mice

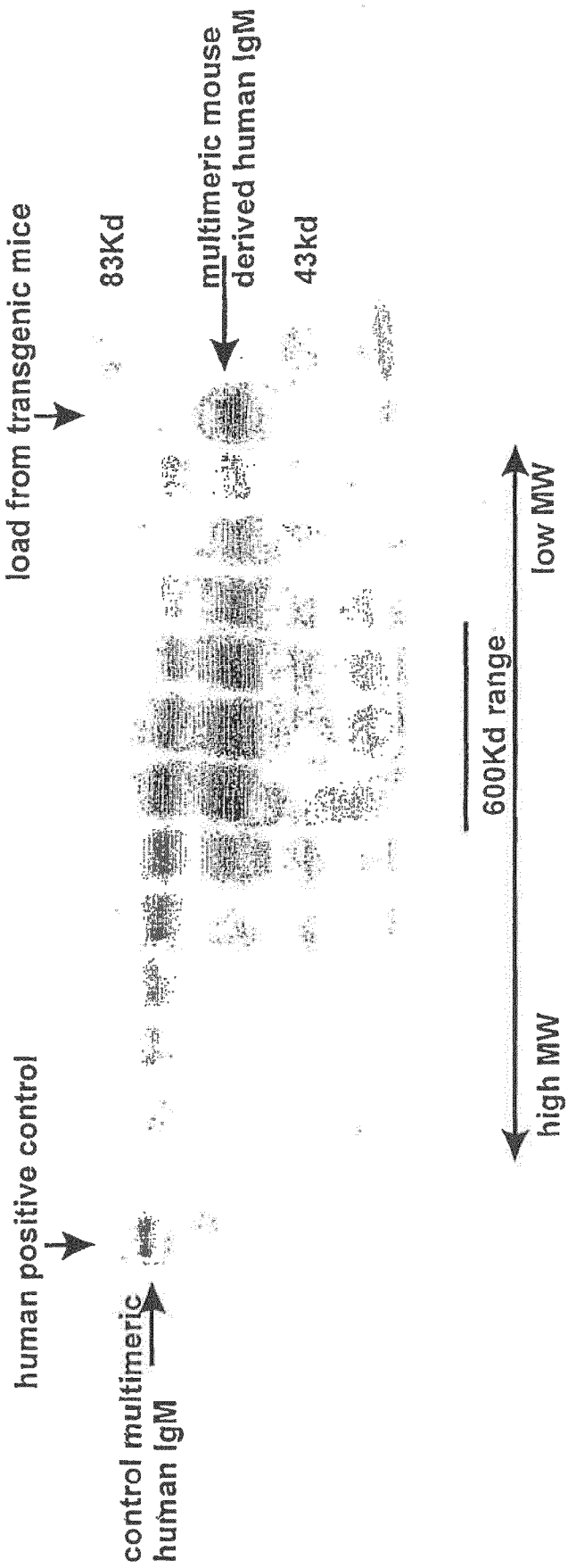
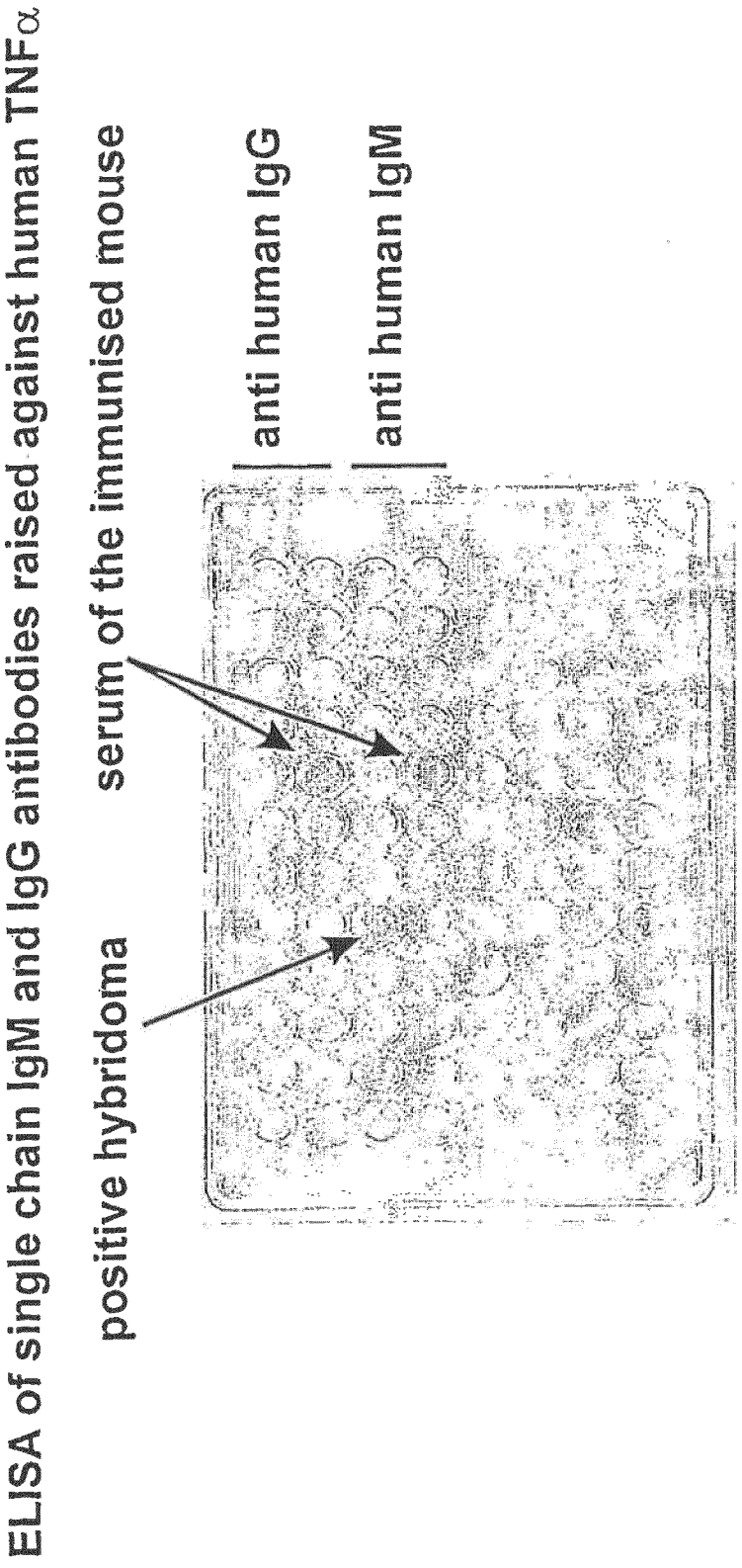


FIG. 16


ELISA of single chain IgM and IgG antibodies raised against human TNFα
positive hybridoma
serum of the immunised mouse
anti human IgG
anti human IgM

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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- WO 02085944 A **[0017] [0047]**
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- WO 2004049794 A **[0029]**
- GB 20030003319 W **[0096]**
- GB 0416392 A **[0137]**
- GB 0511881 A **[0137]**
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- **TACKEN et al.** *J. Immunol.,* 2004, vol. 172, 4934-4940 **[0004] [0006]**
- **GLENNIE ; VAN DER WINKEL.** *Drug Discovery Today,* 2003, vol. 8, 503-5100 **[0004]**
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- **CARTER.** *J. Immunol. Methods,* 2001, vol. 248, 7-15 **[0006]**
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Szabadalmi igénypontok

1. Eljárás V_H csak-nehézlánc ellenanyag előállítására, amely eljárás tartalmazza a következőket:

(a) heterológ V_H nehézlánclókuszt expresszáló transzgenikus patkány immunizálása egy antigénnel, ahol:

(i) a V_H nehézlánclókusz tartalmaz egy olyan variábilis régiót, amely tartalmaz legalább egy természetben előforduló V_H génszegmenst, legalább egy D génszegmenst, legalább egy J génszegmenst és legalább egy nehézlánc konstans régiót, és ahol a V, D és J génszegmensek emberi eredetűek;

(ii) semelyik konstans régió sem kódol C_H1 domént;

(iii) egy V_H génszegmens, egy D génszegmens és egy J génszegmens képes egy VDJ kódoló szekvenciává rekombinálódni;

(iv) a rekombinálódott V_H nehézlánclókusz, amikor expresszálódik, képes olyan oldható csak-nehézlánc ellenanyagot kialakítani, amely tartalmaz egy oldható, antigén-specifikus V_H kötődomént és egy C_H1 doméntől mentes konstans effektor régiót;

(b) egy, a V_H csak-nehézlánc ellenanyagot kódoló nukleinsav-szekvencia izolálása ellenanyag-termelő sejtekből; és

(c) a V_H csak-nehézlánc ellenanyag előállítása rekombináns DNS technikák alkalmazásával.

2. Az 1. igénypont szerinti eljárás, amely tartalmazza a V_H csak-nehézlánc ellenanyag V_H kötődoménjét kódoló V_H lókusz klónozását, valamint a V_H kötődomén bakteriális, élesztőeredetű, emlőseredetű vagy egyéb expressziós rendszerben történő expresszálását is.

3. Az 1. vagy 2. igénypont szerinti eljárás, ahol a transzgenikus patkány úgy lett génmódosítva, hogy csökkent mértékben legyen képes olyan ellenanyagokat termelni, amelyek tartalmaznak könnyűláncokat.

4. Az 1-3. igénypontok bármelyike szerinti eljárás, ahol a patkány endogén immunglobulin nehézlánclókuszai deletálva vannak vagy az expressziójuk gátolva van.

5. Az előző igénypontok bármelyike szerinti eljárás, ahol a V_H nehézlánclókusz több mint egy V_H génszegmenst, több mint egy D génszegmenst és több mint egy J génszegmenst tartalmaz.