EUROPEAN PATENT SPECIFICATION

ANTIGEN BINDING DOMAINS FROM FISH
ANTIGENBINDENDE DOMÂNE AUS FISCH
DOMAINE DE LIASON D'ANTIGENES ISSUS DE POISSONS

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• DOOLEY H ET AL: *Phage display as a tool to investigate antibody repertoire diversity: The novel antigen receptor (NAR) of the nurse shark.* DEVELOPMENTAL & COMPARATIVE IMMUNOLOGY, vol. 24, no. Supplement 1, 2000, pages S37-S38, XP008018904 8th Congress of the International Society of Developmental and Comparative Immunology; Cairns, Australia; July 03-06, 2000 ISSN: 0145-305X

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Description

[0001] The present invention relates to the production of antigen specific antigen binding domains (single domain antibodies) from fish, where the term fish encompasses both cartilaginous (subclass Elasmobranchii) and bony fish (class Osteichthyes). By antigen specific antigen binding domains we mean the variable region of a Novel Antigen Receptor (NAR).

[0002] Antibodies, especially monoclonal antibodies, are useful in, among other things, molecular diagnostics and therapeutics because of their high affinity binding and specificity. However, although it is now relatively simple to produce monoclonal antibodies using animal models the production of human monoclonal antibodies remains difficult. As will be appreciated, when monoclonal antibodies from non-human models are introduced into humans, the body mounts an immune response because the monoclonal antibody is foreign to the human system.

[0003] Recently, it has been appreciated that the activity of the monoclonal antibody can be retained while reducing the rejection thereof in humans by producing single domain antibodies (sdAs) from the variable chain of the relevant antibody. European patent application number 89311731.7 discloses such single domain antibodies and methods for the production thereof in mice.

[0004] Single domain antibodies are also important as they can penetrate tissues taking with them any linked compounds. In addition, they can bind within cavities on the surface of proteins, for example within enzyme binding sites thus disrupting function. Single domain antibodies produced from Camelidae have been shown to recognize protein cavities and as such have the ability to inhibit enzymes (Laurewys et al., EMBO 17 pp3512-3520 1998).

[0005] Although the small size of single domain antibodies produced from Camelidae has allowed the recognition of protein cavities and inhibition of enzyme activity, the range of possible targets may still be relatively low, since many protein cavities may still be too small to be penetrable by single domain antibodies derived from Camelidae.

[0006] WO94/2559 and European patent application number 99200439.0 relate to the production of single domain antibodies from Camelidae heavy chain antibodies. Single domain antibodies produced from Camelidae heavy chain antibodies are more stable than mouse single domain antibodies and can be produced in larger quantities. However, as will be appreciated if, even the smaller members of the Camelidae family, for example llama, are to be kept in humane conditions they require significant areas of land to live upon.

[0007] Diaz M et al., PNAS 24 NOV 1998, vol. 95, no. 24, pages 14343-14348 discloses mutations in the sequences of NAR transmembrane and secretory forms and suggests that hypermutation of NAR does not generate the repertoire, but instead is involved in antigen-driven immune responses.

[0008] Dooley H et al., DEVELOPMENTAL & COMPARATIVE IMMUNOLOGY, vol. 24, no. Supplement 1,2000, pages S37-S38 discloses NAR isolated from a naive nurse shark and the use of phase display to study the functionally active repertoire and determine the mode of antigen binding by individual NAR clones.

[0009] Diaz M et al., INTERNATIONAL IMMUNOLOGY, vol. 11, no. 5, 1999, pages 825-833 analysed 1023 mutations in NAR and found no targeting of the mechanism to any particular nucleotide but did obtain strong evidence for a transition bias and for strand polarity.

[0010] Krishnan U et al., THE GENOME CONFERENCE 2001 - 22nd Annual Conference on the Organisation and Expression of the Genome, 11-15 February 2001 POSTER 1-67, Victoria, Australia discloses that NAR isolated from Wobegong sharks has similar structural features to that found in nurse sharks.

[0011] An object of the present invention is to provide a process for the production of antigen specific antigen binding domains which seeks to alleviate the above problems.

[0012] A further object of the present invention is to provide a composition comprising antigen specific antigen binding domains for the inhibition of protein activity which seeks to alleviate the above problems.

[0013] According to an aspect of the present invention there is provided a process for the production of an antigen specific antigen binding domain using a transformed host containing an expressible DNA sequence encoding the antigen specific antigen binding domain, wherein the antigen specific antigen binding domain is derived from a variable region of the immunoglobulin isotype NAR found in a species of Elasmobranchii subclass wherein the specificity of the antigen specific antigen binding domain is determined by an antigen which is introduced into a member of the Elasmobranchii subclass.

[0014] It has been found that antigen specific antigen binding domains produced from the variable region of NAR found in fish are as stable as single domain antibodies produced from members of the Camelidae family.

[0015] Further many more fish can be kept per unit area than members of the Camelidae family.

[0016] The immunoglobulin isotype now known as NAR (Novel Antigen Receptor), was discovered in the serum of the nurse shark (Ginglymostoma cirratum) as a homodimeric heavy chain complex, naturally lacking light chains (Greenberg et al., Nature 374 pp168-173 1995). However, before the present work by the inventors identification of NAR as an antigen binding domain was not fully appreciated neither was its ability to be raised against a specific antigen.

[0017] Only mammals (humans, mice, rabbits, sheep, camels, llamas, etc.) and some birds (chickens) were believed to be capable of something approaching a secondary immune response such as affinity maturation, antibody class
switching etc. as a response to the presence of foreign antigen. For example, teleost fish (bony), which are much more advanced evolutionarily than sharks, appear to rely solely upon the production of a low affinity, non-specific IgM type response (Watts et al., Aust Vet J 79 pp570-574 2001). A defining characteristic of teleost IgM is their low affinity and ability to non-specifically bind multiple antigens. IgM neutralization is through non-specific multiple binding, resulting mainly in agglutination, etc. Neutralisation without complement is usually associated with specific, high affinity binding and had not until this invention been seen in fish species. The antigen specific antigen binding domains of the present invention have been shown to neutralise activity of an enzyme immunogen directly without calling upon other components of the immune system.

[0018] The NAR variable (V) region conforms to the model of typical Ig superfamily domains with the predicted canonical, intradomain disulphide bond. However, whilst camellid VHH regions have up to 75% sequence identity with other mammalian VH regions, the identity between NAR V and conventional VH domains is as low as 25% (Roux et al., Proceedings of the National Academy of Sciences. USA 95 pp11804-11809 1998).

[0019] Due to this low identity and lack of NAR sequences in the Kabat database, the amino acids of NAR V regions have previously been numbered sequentially (Roux et al., Proceedings of the National Academy of Sciences. USA 95 pp11804-11809 1998). To enable easy comparison of residues in different NAR V molecules, or NAR V region sequences with those of other species during this work, a numbering system was derived for NAR V region based upon that of Kabat et al., (1991) Sequences of Proteins of Immunological Interest, 5th Edition. National Institutes of Health, Bethesda, USA). (Note: this numbering system is used in the Figures attached hereto).

[0020] Immediately apparent from the alignment is the deletion of a large portion of CDR2 (residues 54-65) giving the NAR V region its characteristically small size (see, for example, Figure 2A).

[0021] Initial sequence analysis allowed the classification of NAR V domains into two closely related classes (type I or II), both being constructed from one V, three D and one J segment. Type I regions have non-canonical cysteine residues in CDR2 (C35) and CDR4 (C103), which can form a domain-stabilising disulfide bond. In longer NAR CDR3 loops additional cysteine residue pairs have been observed and almost certainly form disulfide bridges within the CDR, as is found in some cattle VH domains with an unusually long CDR3.

[0022] Type II regions are very similar in overall structure to type I but instead have non-canonical cysteine residues located in CDR1 (C29) and CDR3, which are proposed to form a constraining disulfide bond like that observed in camellid VHH domains. The presence of cysteine within each NAR type is shown in schematic form in Figure 1.

[0023] Recently, an additional NAR type has been identified as the predominant expressed form in nurse shark pups (Type III) but due to its germline joined state displays no juxtoncular diversity.

[0024] In type I and II NAR the DNA encoding the V region is generated by the physical joining of DNA segments which are spatially separate in the genome. This joining process occurs in B-cells and helps generate the diversity of sequence seen for these NAR types. For type III these DNA segments are already physically joined in the DNA of all cells, hence the term germline joined.

[0025] NAR possesses the cluster type genomic organisation usually observed for Ig receptors in cartilaginous fish, but less than five NAR loci are thought to exist, with only two or three being capable of functional rearrangement and expression. The diversity observed in the primary repertoire is generated through recombination mechanisms and, although extensive (due to the presence of three D segments), is localised to CDR3. On encounter with antigen this repertoire is rapidly expanded by extensive mutation. The pattern of mutation in NAR is unlike that observed in shark IgM, which shows low levels of mutation and poor clustering to CDRs, but rather bears the hallmarks of mammalian-like somatic mutation.

[0026] It has recently been found that NAR V is similar to VH, VL and TCR V but distinct from all three, hence its "unique domain architecture". The VH name has been used in the past because the constant portion of NAR is a heavy chain but the V region is actually more like VL/TCR V than VH (i.e. groups closer on a phylogenetic tree). NAR V is not like the camellid VHH domains which are derived from bona fide heavy chain V regions. The antigen binding domain of the NAR is closer to a VL domain naturally lacking VH rather than the other way round.

[0027] The sequence alignment of NAR V and camel VHH clearly shows the huge difference in sequence. If NAR V and camel VHH have the same physical structure (which has been implied but not proven) they achieve this using completely different amino acid sequences, and one would not be able to amplify a NAR V region library using camel VHH library primers. In addition, the ways in which the NAR V and camel VHH gene repertoires are created during VDJ joining are different due to the organisation of the immunoglobulin genes (Schluter et al Immunol Today 18 pp543-549 1997).

[0028] Preferably the transformed host is a prokaryote or a lower eukaryote.

[0029] There are many established prokaryote and lower eukaryote hosts. These hosts are known to correctly express foreign proteins.

[0030] Conveniently the prokaryote host is Escherichia coli.

[0031] In preferred embodiments the expressible DNA sequence is in the form of a phagemid vector.

[0032] Phagemid expression has advantages over phage genome expression in that it results in greater genetic
stability and the bacterial transformation efficiency is higher thus enabling the construction of potentially larger and more diverse libraries.

[0033] To display antibody fragments on phage the gene encoding the variable region of the antibody can be fused to that of a phage surface protein, usually gene III or VIII. Gene III fusion is favoured due to its limited copy number (3-5 copies) on the tip of each phage, minimising possible avidity effects which are undesirable when trying to isolate binders of high affinity. The antibody fragment genes can be cloned directly into the phage genome or fused to gene segments present within phagemid plasmids.

[0034] Preferably the fish is a member of the Elasmobranchii subclass, for example, a shark or a dogfish.

[0035] A greater number of smaller members of the Elasmobranchii subclass can be kept in tanks which are smaller in unit area than the grazing area required for the same number of members of the Camelidae family. As the members of the Elasmobranchii subclass are kept in tanks they can easily be caught for extraction of their blood.

[0036] Conveniently the shark is a nurse shark, *Ginglymostoma cirratum*.

[0037] Preferably the antigen specific antigen binding domain can be targeted to a specific antigen(s).

[0038] Conveniently the antigen specific antigen binding domain is raised to a single antigen.

[0039] According to a further aspect of the present invention there is provided a process for the production of an antigen specific binding domain comprising the steps of:

a) immunising a fish with an antigen;
b) isolating lymphocytes from the fish;
c) isolating RNA for an antigen specific antigen binding domain from the lymphocytes;
d) amplifying DNA sequences encoding the antigen specific antigen binding domain by PCR;
e) cloning the amplified DNA into a display vector;
f) transforming a host to produce a library;
g) selecting the desired clones from the library;
h) isolating and purifying the antigen specific antigen binding domain from these clones;
i) cloning the DNA sequences encoding the antigen specific antigen binding domain into an expression vector;
j) transforming a host to allow expression of the expression vector.

[0040] Screening of displayed libraries for specific binding sites involves repeated cycles of selection with the desired antigen in the process of biopanning. Generally during selection, the library of phage displayed antigen binding domains is incubated with immobilised antigen, unbound phage are washed out and bound phage eluted. This selected population is expanded by bacterial infection and put through further rounds of selection. As each phage encapsulates the DNA encoding the V region it displays, there is a functional linking of genotype and phenotype, reminiscent of membrane bound immunoglobulin on the surface of B-cells. Such cyclic panning has thus proven able to enrich for clones of high affinity, much like *in vivo* antibody selection.

[0041] Preferably before step d) the cDNA of the antigen binding domains is generated.

[0042] Conveniently restriction enzymes are used to digest the amplified DNA sequences encoding the antigen specific antigen binding domain. The restrictions enzymes can be chosen depending upon, for example, the handle of the primers used in the above process.

[0043] In preferred embodiments the restriction enzymes are *NcoI* and *NotI*.

[0044] Conveniently the display vector is any phagemid vector, for example, pHEn2.

[0045] Preferably the expression vector is a soluble expression vector such as pMS100.

[0046] The above vectors are merely examples of the vectors which can be used. It is common general knowledge to those skilled in the art which vectors can be used.

[0047] An antigen specific antigen binding domain produced by the process as defined above is described.

[0048] A composition for the inhibition of protein activity comprising antigen specific antigen binding domains derived from a variable region of the immunoglobulin isotype NAR found in fish is also described.

[0049] Despite the fact that the NAR V region is 12 kDa which is 20% smaller than any 15 kDa single domain antibody derived from *Camelidae*, it was still possible to alter protein activity therewith. Size is a significant factor in the therapeutic applications of antigen specific antigen binding domains and other single domain antibodies, with therapeutic benefits of increased tissue penetration, better access to protein cloths for neutralisation via steric hindrance and reduced immunogenicity, resulting from the use of antigen specific antigen binding domains.

[0050] Antigen specific antigen binding domains derived from NAR therefore have a wider target population than single domain antibodies derived from *Camelidae* by virtue of their smaller size. The potential for immunogenicity is also reduced since in general the smaller the size of a protein the less the immunogenicity.

[0051] Furthermore, although NAR sequences have, in work previous to that of the inventors, been identified at the DNA level, there has been no clue from the DNA evidence that a somatically maturable repertoire, capable of selecting high affinity, specific binders could be a characteristic of the NAR response. Hence, it is unexpected to be able to
generate an NAR library of antigen binding domains derived from sharks and the selection from this of specific and 5
functional antigen specific antigen binding domains and their corresponding receptor genes. Sequencing of these genes
confirms that an atypical (for fish and organisms of this evolutionary lineage) somatically-maturable (showing mutation
from the germ line repertoire) response occurs within the NAR repertoire, driven by the immunisation process. This has
resulted in the selection of highly specific, high affinity antigen binding domains capable of antigen neutralisation in
isolation and not the expected non-specific, low affinity IgM like response typically found in fish and sharks.

Further still, the inventors have been able to isolate NAR antigen specific antigen binding domains and demon-
strate for the first time that the NAR V is able to fold and function in isolation from the rest of the molecule (and in a
non-shark environment), that the antigen specific antigen binding domain matures from the germ line genes to become
specific for antigen (only possible with a library derived from mRNA and not DNA) and that the antigen specific antigen
binding domain is able to bind specifically to the immunising antigen. In summary, as described below, the inventors
have been able to immunize a shark and derive from this immunization a specific, somatically matured antigen specific
antigen binding domain that is of high affinity and specific for the immunogen. In addition, the antigen specific antigen
binding domain is able to neutralise the activity of the immunogen directly, without calling upon other components of
the immune system. According to previous understandings, this should not have been possible for a primitive species
such as sharks.

Inhibition of protein activity is in a concentration dependent manner.
The composition may further comprise a pharmaceutical carrier or diluent therefor.
Such pharmaceutical carriers are well known in the art.

An antigen specific antigen binding domain produced from a variable region of NAR is also described.

The invention will now be described, by way of illustration only, with reference to the following examples and the
accompanying figures.

Figure 1 shows the presence of cysteine amino acid residues within each NAR type, and human, cattle and camel
variable regions for comparison. Canonical cysteines are shown by • and non-canonical cysteines are shown by ●.

Figures 2A, 2B and 2C show the amino acid translations of the sequences obtained in the Examples (SEQ ID. 1 to
51). The sequences are aligned against a typical type I and type II clone sequence (top of each Figure with CDR's
highlighted in bold) dashes indicate identity to the type I clone and * indicates an in-frame stop codon.

Figure 3 shows NAR type I and II variable region amino acid sequence alignment (SEQ 1 and 2). Germline sequence
is given for type I, whilst that given for type II is typical of those observed from somatically mutated cDNA sequences
is indicated by a dash and the CDR's of both sequences are in bold. The numbering above the sequences was
generated by comparison of conserved residues (underlined) with those of other species and is used to enable
comparison of NAR V region sequences.

Figure 4 shows a variability plot for the 29 immune library sequences identified in the Example (pre-selection and
functional). Variability at each position was calculated according to the method of Wu & Kabat (1970) (Journal of
Experimental Medicine 132 pp211-250). The canonical cysteine residues, C22 and 92, are marked by an asterisk.

Figures 5A and B show polyclonal and monoclonal phage ELISA results for selection on Hen egg white lysozyme
(Hel) (Figure 5A) and Chicken ovalbumin (Ova) (Figure 5B). Phage numbers were normalised for each pan prior
to polyclonal analysis. Data presented is a mean of triplicate wells and representative of at least three assays.
Monoclonal results are percentages obtained from 96 clones for each pan.

Figure 6 shows the DNA (SEQ ID. 53 & 54) and encoded amino acid sequence (SEQ ID. 52) of the α-HEL 5A7
clon. CDRs are highlighted in bold.

Figure 7 shows the DNA (SEQ ID. 56 & 57) and encoded amino acid sequence (SEQ ID. 55) of the α-HEL 4F11
clon. CDRs are highlighted in bold.

Figure 8 shows the amino acid alignment of the two α-HEL clones, 5A7 (SEQ ID. 52) and 4F11 (SEQ ID. 55), with
a typical type I clone (SEQ ID. 1). Sequences are numbered according to Figure 3 for ease of comparison, differences
between the selected clones are highlighted in underlined and CDR's are highlighted in bold, * conserved residues
in all sequences, • conserved substitutions, semi-conserved substitutions.
Figure 9 shows the DNA (SEQ ID. 59 & 60) and encoded amino acid sequence (SEQ ID. 58) of the α-Ova 4H11 clone. CDRs are highlighted in bold.

Figure 10 shows the DNA (SEQ ID. 62 & 63) and encoded amino acid sequence (SEQ ID. 61) of the α-Ova 3E4 clone. CDRs are highlighted in bold.

Figure 11 shows amino acid alignment of the two α-Ova clones, 4H11 (SEQ ID. 59) and 3E4 (SEQ ID. 61), with a typical type I clone (SEQ ID. 3). Sequences are numbered according to Figure 3 for ease of comparison, differences between the selected clones are underlined and the CDR's are highlighted in bold. * conserved residues in all sequences; _ conserved substitutions, semi-conserved substitutions.

Figure 12 shows binding analysis of α-HEL clone 5A7. Serial dilutions of crude periplasmic release solution were applied to an ELISA plate coated with each of the test proteins at 10 μg/ml and blocked with Marvel. Data presented is a mean of triplicate wells and representative of at least three repeat assays.

Figure 13 shows binding analysis of α-HEL clone 4F11. Serial dilutions of crude periplasmic release solution were applied to an ELISA plate coated with each of the test proteins at 10 μg/ml and blocked with Marvel. Data presented is a mean of triplicate wells and representative of at least three repeat assays.

Figure 14 shows binding analysis of α-Ova clone 4H11. Serial dilutions of crude periplasmic release solution were applied to an ELISA plate coated with each of the test proteins at 10 μg/ml and blocked with Marvel. Data presented is a mean of triplicate wells and representative of at least three repeat assays.

Figure 15 shows a comparison of the stability of the anti-HEL clones 5A7 and 4F11 to irreversible thermal denaturation. Data presented is a mean of triplicate wells and representative of at least three repeat assays.

Figure 16 shows a lysozyme enzymatic inhibition assay. Purified HEL-5A7 NAR V region protein at a final concentration of 2500 nM (filled circle), 250 nM (open triangle) or 25 nM (filled square) were pre-incubated with HEL prior to the introduction of M. lysodeikticus bacterium. The control wells (open diamond) contained buffer in place of HEL-5A7 protein. The data presented is an average of 3 replicates and a typical data set from three repeat experiments.

**Example**

**Bacterial strains**

**[0058]** The electroporation-competent strain *E. coli* XL1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacZAM15 Tn10 (TeR)] ] (Stratagene Ltd.) was used to prepare and pan the NAR V region phage display libraries.

**PCR materials**

**[0059]** All custom oligonucleotides used throughout this work were ordered from Sigma-Genosys Ltd., and were desalted and/or HPLC purified. Library primer sequences were as follows (all 5' to 3'):

F1 For1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAR F4 For1</td>
<td>ATA ATC AAG CTT GCG GCC TTC ACA GTC ACG</td>
<td>(SEQ ID. 64)</td>
</tr>
<tr>
<td>NAR F4 For2</td>
<td>ATA ATC AAG CTT GCG GCC TTC ACA GTC ACG</td>
<td>(SEQ ID. 65)</td>
</tr>
<tr>
<td>NAR F1 Rev</td>
<td>ATA ATC AAG CTT ATG CTA GCG CGA GTG GAC CAA</td>
<td>(SEQ ID. 66)</td>
</tr>
</tbody>
</table>

**[0060]** All PCR reactions were performed on a Hybaid PCR sprint block in Hybaid 0.2 ml thin-walled omnitubes.
Construction of NAR V region libraries for phage display

RNA preparation

[0061] To enable production of the immune library, three nurse sharks were immunised five times with Hen egg-white lysozyme (HEL) (over a period of approximately 8 months). Blood samples were taken from each shark following each immunisation, peripheral blood lymphocytes isolated, and total RNA prepared for each bleed. The RNA from bleeds 4 and 5 for each of the three sharks was pooled and stored at -80°C until required for cDNA synthesis.

cDNA synthesis and PCR amplification

[0062] For cDNA synthesis, ready-to-go RT-PCR beads (200 μM each dNTP, 10 mM Tris-HCl buffer, 60 mM KCl 1.5 mM MgCl₂, M-MuLV reverse transcriptase, RNase/DNase free BSA and 2 U Taq DNA polymerase) (APB Ltd.) were reconstituted in 45 μl of DEPC treated H₂O by incubating on ice for 5 min or until the beads were completely dissolved. To each tube 2 μl of nurse shark tRNA at 2 μg/μl and 2 μl of NAR F4 For primer or F4 For2 primer at 25 μM/μl were added. Both of these primers are specific for NAR framework region 4 and have a NotI site incorporated in the handle to allow subsequent cloning into the phagemid vector. Tubes were flicked gently to mix contents and incubated on a PCR block pre-warmed to 46°C for 30 min. Following cDNA synthesis, tubes were incubated at 95°C for 7 min to inactivate the reverse transcriptase and denature the template.

[0063] To each tube 2 μl of the common primer NAR F1 Rev at 25 pM/μl, containing a Ncol site in its handle was added, tubes were pre-heated to 95°C and 1 μl of Taq DNA polymerase at 1 U/μl added to each prior to cycling 32 times at 95°C for 2 min, 55°C for 1 min and 72°C for 1 min 30 s.

[0064] Following PCR amplification type I and type II, products were PAGE purified on a 1.5% gel a strong band was visualised at approximately 400 bp for both primer sets indicating successful amplification of the NAR V region.

Cloning of NAR V region into the phagemid vector pHEN2

[0065] PAGE-purified PCR product was digested with NcoI and NotI restriction enzymes, at the sites incorporated by the handled primers used for amplification, to allow cloning into the phagemid vector pHEN2. Restricted DNA was purified on a 1.5% agarose gel and the DNA excised and cleaned.

[0066] Plasmid DNA, harvested from an overnight culture of E. coli XL1-Blue and phenol: chloroform treated, was similarly cut with NcoI and NotI restriction enzymes. Double-cut vector was purified on a 0.7% agarose gel and DNA extracted. For library construction digested vector was not treated with calf alkaline phosphatase.

[0067] To enable quantification, 2 μl of suitably digested PCR product and pHEN2 vector were run on a 1% agarose gel against 2 μl of DNA marker VI (Boehringer Ltd.) and band intensities evaluated by eye to judge relative amounts of DNA present. Ligation were performed with equal amounts of vector and insert DNA in the presence of 2.5 μl of 10 x ligase buffer and 1 μl of T4 ligase. The final volume was made up to 25 μl with H₂O₂ and incubated overnight at 15°C. For library construction 30-40 such ligations were performed.

[0068] Following incubation overnight, ligation products were pooled, phenol:chloroform cleaned and the resultant DNA pellet reconstituted in approximately 100 μl of 1:10 dilution of 10 mM Tris-HCl, pH 8.5. DNA was then ready for transformation into electroporation-competent cells.

Transformation of electroporation-competent cells and evaluation of the resultant library

[0069] Ligated DNA was aliquotted into chilled electroporation cuvettes and to each 40 μl of freshly thawed electroporation-competent XL1-Blue cells was added. Cells were electroporated and resuspended in 100 μl ice-cold 2xTY media with 1% glucose (w/v) added. Dilutions at 10⁻², 10⁻⁴ and 10⁻⁶ were performed for each transformation and plated on TYE agar containing 100 μg/ml ampicillin and 1-2% glucose (w/v). The remaining bacterial suspension was plated straight onto 140 mm petri-dishes containing TYE with ampicillin and glucose (as above). All plates were grown overnight at 37°C.

[0070] Following incubation overnight, colonies from the dilution plates were counted to give an estimate of the final library size, approximately 5 x 10⁶ members. Approximately 100 individual colonies were PCR screened using 1 μl each of the primers LM35 (5’ CAGGAAACACCTATGAC 3’) (SEQ ID. 69) and pHEN seq (5’ CTATGACGCCCATCTCA 3’) (SEQ ID. 70) at 25 μM/μl, 1 μl of dNTPs at 25 μM each, 2 μl of 50 mM MgCl₂, 5 μl of 10 x Taq polymerase buffer, 1 μl Taq polymerase (at 1 U/μl) and 39 μl Sterpaks H₂O. PCR was undertaken as follows; 1 cycle at 95°C for 3 minutes (to lyse bacteria) and 20 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. PCR product was run on a 1.5% agarose gel containing EtBr against molecular weight marker VI (Boehringer Ltd.) to evaluate the percentage of the library carrying NAR V region insert. Using this method, 75% of the library was observed to be carrying an insert
approximating that expected for the NAR V region, giving a functional library size of 3.75 x 10^6 members. Fifty clones, established in this way to be carrying correctly sized inserts, were then sequenced to evaluate library diversity.

[0071] The encoded amino acid translations of the sequences obtained are shown in Figures 2A, B and C.

[0072] Of the 50 clones sequenced, 6 were found to harbour one or more stop codons encoded by an in-frame TGA codon within CDR3. In the case of clones 13 and 19 the stop codon is probably a consequence of the D3 and D2 segments (respectively) being utilised in a non-preferred reading frame (Roux et al., Proceedings of the National Academy of Sciences, USA 95 pp11804-1180 (1998)). The reason for the stop codons in the other 5 clones is less distinct but is likely due to somatic hypermutation within this region.

[0073] A further 15 clones carried frameshift mutations leading to the production of non-sense or truncated proteins. For the majority of these clones the frameshift occurred within CDR3, possibly as a consequence of nucleotide addition or deletion during the recombination process. For clones 14 and 41 the frameshift mutation arose within Fr2 (position 41 according to Figure 3) and Fr3 (position 67) respectively and are more likely due to polymerase errors during library construction (the frameshift in clone 14 occurs immediately after a long poly-A tract in the DNA sequence).

[0074] The sequence alignment and the variability plot of the 28 clones encoding functional inserts (Figures 2 and 4) show good diversity, with each clone having a unique amino acid sequence. Variability is seen to be focussed across CDR3 which, like clones from a similarly constructed naive library, varied greatly in both sequence and length. The immune nature of the library is important as NAR V regions which bound to antigen could not be isolated from a naive library (i.e. without prior immunisation).

[0075] Both NAR types were represented, with approximately 80% being type I and 20% type II, however a number of clones proved difficult to assign to an NAR type. For example, clone 33 has a type II Fr1 but type I CDR3 and Fr4, whilst clones 06, 40 and 46 have a type I Fr1 and CDR3 but a type II Fr2 and Fr4. This finding suggests the possibility that gene conversion may be occurring between the NAR genes.

[0076] A number of other clones also show some atypical features which were not observed with the naive library pre-selection clones. Clones 24 and 36 are both assigned as type I on the basis of other sequence characteristics but do not carry the pair of cysteine residues normally observed in the type I CDR3. The clones 06, 40, 46 and 48 all encode an uneven number of cysteine residues. As mentioned previously in the case of 06, this may be due to gene conversion. Very few clones bearing an uneven number of cysteines have been observed previously and so it is thought that the V region must be under considerable pressure to maintain an even number of cysteine residues, enabling formation of disulphide bonds. The consequence of unpaired cysteines within the NAR V region is, as yet, unknown but may be detrimental to domain folding. If this is indeed the case then such clones will probably be eliminated from the library during early pans due to their toxicity to the expressing bacteria.

[0077] Clone 02 encodes 4 cysteine residues in its CDR3, giving this V region a total of 8 cysteine residues and the potential to form 4 disulphide bonds. Such type I domains carrying 4, or occasionally 6 or more, cysteine residues have been previously encountered. The ability to form these additional disulphide bonds, combined with the small size of the NAR V region, may provide an additional source for highly stable antibody fragments.

[0078] Colonies, which were not sequenced, were scraped from the library plates with a sterile spreader into a final volume of 10 ml 2xTY medium containing 100 µg/ml ampicillin and 2% glucose. Cells were combined with sterile glycerol to 20% (v/v), and following thorough mixing aliquotted as 500 µl shots and flash-frozen prior to storage at -80°C.

**Panning of NAR V region library against protein antigens Growth of the library**

[0079] A single aliquot of library stock was added to 200 ml of pre-warmed 2xTY medium containing ampicillin at 100 µg/ml and 1-2% glucose (v/v) and grown at 37 °C/250 rpm until log phase (OD_600 of 0.4 - 0.8) was reached. To a 50 ml sample taken from the culture approximately 10^{15} of M13K07 helper phage were added and the culture incubated at 37 °C without shaking to allow infection. Following incubation the culture was spun at 3.5K rpm/ 4 °C for 10 min and the cell pellet re-suspended in 100 ml of 2xTY containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1-0.25% glucose and incubated overnight at 30 °C/250 rpm to allow library expression and rescue.

[0080] The overnight culture was spun at 12K rpm/ 4 °C for 20 min, 80 ml of supernatant was removed and added to 20 ml of PEG/NaCl, mixed well and incubated on ice for at least 1 h. The precipitated phage was pelleted at 12K rpm/ 4 °C and resuspended in 2 ml PBS. The phage suspension was spun at 13K rpm for 10 min to remove any remaining bacterial debris and the phage supernatant stored at 4 °C. The phage stock was titrated by performing serial dilutions in PBS and the addition of 900 µl of a log phase culture to 100 µl of each dilution. Following incubation at 37 °C for 30 min, 100 µl of each dilution was plated on TYE plates containing ampicillin at 100 µg/ml and 1% glucose and incubated overnight at 37 °C. The phage titre could be estimated by counting the resulting colonies.

**Library selection**

[0081] Nunc Maxisorp Immuno test tubes (Gibco BRL, Life technologies Ltd.) were coated with either HEL or Ova in
4 ml of PBS overnight at 4 °C. The tube was then washed 3 times with PBS before being blocked with 2% Marvel in PBS (MPBS) for 2 h at room temperature, following which it was washed a further 3 times with PBS. Selection was conducted by incubating the coated immunotube for 1 h at room temperature with 1 ml of phage stock in 3 ml of 2% MPBS on an over-and-under tumbler. A further hour of stationary incubation was allowed before the supernatant containing unbound phage was discarded and bound phage eluted as described below.

**Elution and rescue of antigen-bound phage**

**Triethylamine elution**

[0082] Binding individuals of the antigen specific antigen binding domain library, displayed on the phage strain M13K07, were eluted using the alkali triethylamine.

[0083] Following incubation with phage the immunotube was washed 20 times with PBST, excess liquid drained off and 1 ml of 100 mM triethylamine added. The tube was then rotated for a maximum of 10 min at room temperature to elute bound phage. Following incubation the phage solution was neutralized by mixing with 500 µl of 1 M Tris-HCl. In this state the phage solution was stored at 4 °C for further use (or long-term at -20 °C if glycerol added at 15% v/v).

[0084] To 750 µl of the triethylamine-eluted phage 10 ml of a log phase bacterial culture was added and the culture incubated at 37 °C without shaking for 30 min. Serial dilutions of the culture were prepared in 2xTY and plated on TYE plates containing 100 µg/ml ampicillin and 2% glucose to allow the number of rescued phage to be estimated. The remaining infected culture was spun for 10 min at 13K rpm, re-suspended in 100 µl of 2xTY and plated on a 140 mm petri-dish containing TYE as above. Plates were grown overnight at 37 °C.

**Rescue of selected phage**

[0085] After overnight growth, colonies were scraped from the large petri-dishes into 2 ml of 2xTY medium with a sterile scraper and the suspension mixed thoroughly. Following inoculation of 50 ml 2xTY containing 100 µg/ml ampicillin and 1-2% glucose with 50 µl of this suspension, 1 ml of the remaining bacteria was mixed with 15% glycerol (v/v) and stored at -80 °C as a stock. The 50 ml culture was incubated at 37 °C/250 rpm until the OD_{600} reached 0.4, whereupon 15 ml was removed, added to approximately 10^{10} helper phage and incubated for 30 min at 37 °C. Following incubation the culture was spun at 3.5 K rpm for 10 min and the resultant cell pellet re-suspended in 2xTY containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1-0.25% glucose and incubated overnight at 30 °C/250 rpm.

[0086] The overnight culture was spun at 12K rpm for 10 min and 40 ml of supernatant added to 10 ml of PEG/NaCl, and mixed well prior to incubation on ice for at least 1 h. The phage pellet was again re-suspended in 2 ml of PBS and spun for 10 min at 13K rpm to remove any remaining bacterial debris and the phage stored at 4 °C for the short term.

[0087] Further rounds of selection were carried out with phage rescued from the previous round of selection, as above, on antigen coated immunotubes.

[0088] The immune library was subject to five rounds of panning against the protein antigens Hen egg white lysozyme (HEL) and Chicken ovalbumin- (Ova), independently, using M13K07 helper phage and triethylamine elution. A summary of the panning results are given in Table 1.

[0089] In an attempt to minimize loss of clone diversity in early rounds of selection the antigen coating density was kept constant at 100 µg/ml for pans 1 and 2. Following the first round of panning approximately 10^{6} phage were eluted from both the HEL and Ova coated immunotubes, increasing 10-fold following pain 2. For pans 3 and 4 the antigen coating density was reduced for each pan in an attempt to select higher affinity binders. Whilst the number of phage eluted following HEL selection remained constant at ~10^{8} for both pans that for Ova selection dropped to 10^{3} in pan 3, rising back to 10^{5} following pan 4. For pan 5 the antigen coating concentration was further reduced and selection was accompanied by a significant drop in the number of phage eluted. Due to this reduction in the number of phage eluted polyclonal and monoclonal phage ELISAs were conducted to determine if enrichment of HEL or Ova binders was occurring (Figure 5).

[0090] The binding of the HEL-selected polyclonal phage showed a small increase in OD_{600} over pans 1 and 2, with a significant increase following pan 3. A further small increase in signal followed pan 4, but afterwards pan 5 dropped back to the level observed for earlier pans. A similar pattern was observed for the Ova-selected polyclonal phage with the highest binding being obtained for phage rescued after pan 4, however in this instance the OD_{600} values remain low (below 0.25) for all pans.

[0091] Monoclonal phage ELISAs show an increase in the number of positive phage for both sets of selection over pans 1 to 4. In the case of HEL selection this increase was from less than 1% to approximately 80% following pan 4. For Ova selected clones the numbers of positives was slightly lower but regardless increased from less than 1% to approximately 66% after the fourth pan. Following pan 5 the number of HEL-positive clones remained constant at 80% but the number of Ova-positive monoclonals dropped back to the levels observed in earlier pans (~10%).
The drop in the number of clones able to bind Ova after pan 5 indicates that for this pan the protein coating concentration has been reduced such that the selection is too stringent and the majority of clones are no longer able to bind. No such drop is observed for the HEL-selected monoclonal assay, indicating that the affinity of these clones for their antigen is probably higher. This shows that the antigen specific antigen binding domains produced by the sharks are very specific as the sharks were immunised with HEL and only HEL binders could be isolated, Ova data shows no binders. For this reason a selection of clones from pans 3 and 4 were sequenced for Ova but from pans 4 and 5 for HEL.

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Selection analysis

Polyclonal phage ELISA

A 96-well Immulon 4 ELISA plate (Dynatech Laboratories Ltd.) was coated with 100 µl of antigen at 10 µg/ml for 1 h at 37°C. Following three washes with PBST the wells were blocked with 300 µl of 2% MPBS (PBS with 2% w/v marvel added) for a further hour at room temperature of overnight at 4°C. Wells were washed 3 times with PBST and to individual wells 10 µl of PEG precipitated phage from each pan, in 100 µl of 2% MPBS, was added and the plate incubated for 1 h at room temperature. The phage solution was discarded and the plate washed with PBST 3 times. To each well 100 µl of anti-M13 monoclonal HRP conjugate (APB Ltd), diluted 1 in 5000 in PBS, was added and incubated at room temperature for 1 h. The plate was washed 5 times with PBST and developed with 100 µl per well of TMB substrate, the reaction stopped with 50 µl per well of 1 M H_{2}SO_{4} and the plate read at 450 nm.

Monoclonal phage ELISA

Individual colonies growing on TYE plates were picked into 100 µl 2xTY medium containing 100 µg/ml ampicillin and 1-2% glucose on a sterile 96-well ELISA plate, for each of the pans, and grown overnight at 37°C/250 rpm. Following growth, a 96-well transfer device was used to inoculate a fresh 96-well plate containing 200 µl per well of 2xTY with 100 µg/ml ampicillin and 1-2% glucose. Bacteria were grown for 2 h at 37°C/250 rpm. To the original overnight plate glycerol was added to give a final concentration of 15% and the plates stored at -80°C as a bacterial stock.

After the two hour incubation 25 µl of 2xTY containing 100 µg/ml ampicillin, 1-2% glucose and 10^{10} helper phage were added to each well. The plate was then incubated for a further hour at 37°C/250 rpm before being spun at 2K rpm for 10 min to pellet the bacteria. Supernatant was aspirated from the plate and the resultant pellet re-suspended in 200 µl 2xTY containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and glucose at 0.25% (w/v). The plate was then incubated overnight at 30°C/250 rpm.

The overnight plate was spun at 2K rpm for 10 min to give a supernatant containing monoclonal phage supernatant. To suitably coated and blocked plates, 50 µl of this phage supernatant in 50 µl of MPBS was added per well and the plate incubated at room temperature for 1 h. Following incubation the plate was incubated with anti-M13 HRP conjugated antibody and developed as normal.
Subcloning and sequencing of positive monoclonal phage clones

Following determination of individual clones giving a positive signal for antigen binding, 5 ml of 2xTY containing 2% glucose and 100 µg/ml ampicillin was inoculated from the appropriate clone source. Taking into account the results of the monoclonal phage ELISAs fifteen HEL-positive clones were picked at random from pans 4 and 5, whilst those for Ova were picked from pans 3 and 4. Following overnight incubation of the cultures at 37°C/250 rpm plasmid was prepared as set out above. A 20 µl sample of plasmid was then digested with the restriction enzymes NcoI and Ncol and the -400 bp fragment corresponding to the NAR V region fragment PAGE purified and recovered. Purified V region fragments were then ligated into similarly cut, alkaline phosphatase treated and cleaned pMS100 expression vector. Following overnight incubation at 15°C the resultant vector, harbouring the NAR V insert fused upstream of the HuCk domain and 6His tail, was transformed into electroporation-competent E. coli XL1-Blue cells. Colonies were picked, grown as overnight cultures in 5 ml TB (containing 2% glucose (v/v), 100 µg/ml ampicillin, 25 µg/ml tetracycline) and glycerol stocks and plasmid prepared.

Inserts were sequenced from plasmid using the M13 reverse (5’ TTCACACAGGAAACAG 3’) (SEQ ID. 67) and HuCk forward (5’ GAAGATGAGACAGATGTG 3’) (SEQ ID. 68) primer. Once sequence data had been generated the clone was given a unique name to enable identification.

On translation only two different sequences were obtained from the 15 HEL-selected clones and two from the 15 Ova-selected clones.

The clones 5A7 and 4F11 were chosen to represent the two different amino acid sequences found within the HEL-selected positive clones (Figures 6 and 7). The two clones are both conventional NAR type I, and so are illustrated aligned against a typical type I clone in Figure 8. The two clones differ from one another at only two positions (43 & 44), both lying within Fr2 and carry identical CDR3 regions. The clones 4H11 and 3E4 were chosen to represent the two different amino acid sequences found within the Ova-selected positive clones (Figures 9 and 10). Again these clones were both conventional NAR type I and as such are shown aligned against a typical type I clone in Figure 11. These clones differ at 6 amino acids; three within Fr1 (positions 13, 14 & 30), two within Fr2 (positions 46 & 47) and one within CDR3 (position 101).

Expression of antigen binding domains in E. coli

Large scale expression

A single colony of transformed E. coli was used to inoculate 5 ml LB containing 1% glucose (v/v), 12.5 µg/ml tetracycline and 50 µg/ml ampicillin and grown up at 37°C /250 rpm overnight. This culture was used to seed 50 ml TB medium containing 1% glucose (v/v), 12.5 µg/ml tetracycline and 50 µg/ml ampicillin in 250 ml baffled flasks, at 1% v/v. The 50 ml cultures were grown over a period of 24 hours at 25°C/250 rpm, with one change of media after approximately 10 hours growth. Growth of all the cultures was good with the overnight OD600 being in the order of 10-20 OD units.

Overnight cultures were pelleted at 4 K rpm /4°C for 20 min. Pellets were resuspended in 50 ml fresh TB containing 50 µg/ml ampicillin and given 1 h at 25°C /250 rpm to recover before induction with 1.5 mM IPTG for 3.5-4 h and release of periplasmic contents.

Periplasmic burst release method

The cell pellet resulting from centrifugation was resuspended in 10% of the original culture volume of fractionation buffer (100 ml 200 mM Tris-HCl, 20% sucrose, pH 7.5, 1 ml 100 mM EDTA/L of culture). The suspension was incubated on ice with gentle shaking for 15 min following which an equal volume of ice-cold sterile H2O was added and incubation continued for a further 15 min (method modified from French et al., Enzyme & Microbial Technology 19 pp332-338 1998). The suspension was spun at 13K rpm / 4°C for 20 min, the supernatant containing the periplasmic fraction harvested and passed through a 0.22 µm filter (Sartorius Instruments Ltd.).

None of the cultures showed any sign of bacterial lysis during the 4 h induction period and expression yields in the order of 1 mg crude NAR protein per litre of culture were obtained. In this example the protein expressed from the four selected clones was IMAC purified via the 6His tail.

ELISA analysis of antigen binding domains

Antigen binding ELISA

An Immulon 4 96-well flat bottomed ELISA plate was coated with a suitable concentration of the desired antigen at 100 µl per well and the plate incubated at 37°C for 1 h. The plate was washed 3 times with PBST prior to blocking
with 200 µl per well of PBS containing 2% Marvel (w/v) for 1 h at 37°C. Wells were washed a further three times with PBS before addition of samples.

[0106] A 1 in 5 dilution of crude periplasmic release solution was prepared, added to the top wells of the plate at 200 µl per well and doubling dilutions in PBS performed. Plates were then incubated at 4°C for 1 h. Each plate was washed a further 5 times with PBS. Goat anti-HuCk peroxidase conjugate antibody was diluted 1:1000 in PBS and 100 µl added to wells containing antigen binding domains. Plates were incubated for 1 h at 4°C and following 6 washes with PBS the ELISA was developed as described previously and the plate read at 450 nm.

[0107] The HEL-selected clone 5A7 (Figure 12) shows good binding to HEL at the top dilution applied and as the sample is serially diluted binding reduces accordingly. Limited binding to the highly related protein turkey egg-white lysozyme (TEL) is observed at the highest dilution but no binding is observed to the proteins Chicken ovalbumin (Ova), Bovine serum albumin (BSA), Keyhole limpet haemocyanin (KLH) or the blocking agent Marvel. An identical pattern of protein binding is also observed for the HEL-selected clone 4F11 (Figure 13), which is not surprising considering the high degree of amino acid sequence similarity between these two clones (111/113 aa identical). The OD450 signals obtained for 3F11 are slightly higher than those for 5A7, but this may simply be due to small differences in the amount of protein present in the samples.

[0108] The Ova-selected clone 4H11 (Figure 14) showed no binding to any of the proteins tested, including Ova, the antigen it was selected against. To ensure that this was not simply a consequence of there being too little protein present in the assay, a binding assay was performed with undiluted periplasmic release solution. In this instance some binding to all of the proteins was observed for the wells containing the top dilutions of 4H11 protein. This binding was immediately lost once the sample was diluted and so is likely to be non-specific, no doubt resulting from very high concentrations of protein being present. This data supported the initial finding that the 4H11 clone does not bind significantly to Ova. The 3E4 clone, like 4H11, does not show binding to the proteins HEL, BSA, KLH, TEL or the blocking agent Marvel, however low level binding is observed for this clone to the selection antigen Ova. The pattern of binding by this clone to Ova is unusual in that binding at the highest protein concentration is low and shows no significant drop on dilution of the sample. When the protein concentration was increased by repeating the assay with undiluted periplasmic solution a similar pattern of binding was observed, thus negating the possibility that the protein concentration was initially too low. The reason for this unusual binding is as yet unknown, but may be due to 3E4 binding only with low affinity to Ova.

[0109] The distinct lack of NAR clones capable of binding antigen in a library previously constructed from material from a naïve animal and the isolation of HEL-binding, but not Ova-binding clones, from the library constructed from the HEL immunised animals illustrates the highly specific nature of the NAR response following antigen challenge. In other words, antigen specific antigen binding domains with a specific specificity are produced.

Stability analysis of selected clones

[0110] As clones 5A7 and 4F11 were shown to be capable of binding HEL in the antigen binding ELISA it was possible to test the stability of these clones to thermal denaturation. Sub-saturating dilutions of both of the clones, ascertained from the antigen binding curves, were prepared and incubated at a range of temperatures for 3 h prior to their addition to a HEL coated ELISA plate. The samples were then incubated on the ELISA plate for an hour at 4°C and binding detected with an anti-HuCk HRP conjugated antibody. Stability of the antigen binding domains was plotted as a percentage of that obtained for a control sample which had not been heat treated (Figure 15).

[0111] Both clone 5A7 and clone 4F11 show considerable resistance to irreversible denaturation losing 50% functionality at approximately 85°C and retaining approximately 30% functionality after 3 h at 95°C. This high stability is probably a consequence of the additional, non-canonical cysteine residues found within the NAR V domain. Both clones encode 6 cysteine residues and therefore are capable of forming 3 intradomain disulphide bonds, which (if formed) would contribute greatly to the high stability of these domains. The shape of the stability curves for both of the clones is almost identical and the minor difference in stability between the clones may be simply due to assay variability.

[0112] Repetition of this assay utilising an anti-His HRP conjugated antibody to detect binding generated values which were not significantly different to those obtained with the anti-Huck secondary antibody, indicating the drop in signal is caused by reduced binding of the NAR V domains, due to denaturation, and not simply reduced detection via the HuCk tag.

Inhibition of protein activity

[0113] The ability of HEL-5A7 to inhibit the enzymatic activity of HEL was tested by mixing 12.5 µl of HEL with 12.5 µl of purified HEL-5A7 protein in a sterile 96 well tissue culture plate, to give a final HEL concentration of 10 µg/ml and HEL-5A7 concentrations of 2500 nM, 250 nM and 25 nM. The control well was set up with buffer replacing HEL-5A7. A sample of freeze dried Micrococcus lysodeikticus was reconstituted in 0.1 M phosphate/citrate buffer (pH 5.8) containing 0.09% NaCl, mixed thoroughly and 175 µl added to the prepared wells. The plate was read over a period of 30 min (at 1 min intervals) at 450 nm. Enzymatic activity was plotted as percentage initial absorbance against time for each sample.
The introduction of HEL-5A7 protein to the assay reduced the rate of cell lysis in a concentration dependent manner with respect to the control (Figure 16). With HEL-5A7 protein at a final concentration of 2500 nM the rate of cell lysis (9.3x10^{-9} OD units/min) is almost halved when compared to the control (17x10^{-9} OD units/min) indicating that the HEL-5A7 region binds within or adjacent to the lysozyme active site cavity. A similarly prepared antigen specific antigen binding domain raised against an unrelated antigen showed no effect upon the rate of cell lysis when introduced to the assay at the same concentrations.

It will be understood that the embodiment illustrated shows one application of the invention only for the purposes of illustration. In practice the invention may be applied to many different configurations, the detailed embodiments being straightforward for those skilled in the art to implement.

SEQUENCE LISTING

Aberdeen University University of Maryland

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Ginglymostoma cirratum

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Thr Val Asn
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Ala Val Thr Val Asn
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Ser Phe Ser Leu Thr Ile Asn Asp Leu Thr Val Glu Asp Gly Gly Thr
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1  5  10  15

Ala

Claims

1. A process for the production of an antigen specific antigen binding domain using a transformed host containing an expressible DNA sequence encoding the antigen specific antigen binding domain, wherein the antigen specific antigen binding domain is derived from a variable region of the immunoglobulin isotype NAR found in a species of *Elasmobranchii* subclass wherein the specificity of the antigen specific antigen binding domain is determined by an antigen which is introduced into a member of the *Elasmobranchii* subclass.

2. A process according to claim 1 wherein the transformed host is a prokaryote or a lower eukaryote.

3. A process according to claim 2 wherein the prokaryote host is *Escherichia coli*.

4. A process according to any preceding claim wherein the expressible DNA sequence is in the form of a phagemid vector.

5. A process according to any preceding claim wherein the species of *Elasmobranchii* subclass is a shark or a dog fish.

6. A process according to claim 5 wherein the shark is a nurse shark.

7. A process according to any preceding claim wherein the antigen specific antigen binding domain is targeted to a specific antigen(s).

8. A process according to any preceding claim wherein the antigen specific antigen binding domain is raised to a single antigen.

9. A process for the production of an antigen specific antigen binding domain comprising the steps of:

a) immunising a member of the *Elasmobranchii* subclass with an antigen;
b) isolating lymphocytes from the member;
c) isolating RNA from the lymphocytes;
d) amplifying DNA sequences encoding the antigen specific antigen binding domain by PCR;
e) cloning the amplified DNA into a display vector;
f) transforming a host to produce a library;
g) selecting the desired clones from the library;
h) isolating and purifying the antigen specific antigen binding domain from these clones;
i) cloning the DNA sequences encoding the antigen specific antigen binding domain into an expression vector;
j) transforming a host to allow expression of the expression vector.

10. A process according to claim 9 wherein before step d) the cDNA of the antigen specific antigen binding domain is generated.

11. A process according to either claim 9 or 10 wherein restriction enzymes are used to digest the amplified DNA sequences encoding the antigen specific antigen binding domain.

12. A process according to claim 11 wherein the restriction enzymes are Ncol and Notl.

13. A process according to any of claims 9 to 12 wherein the display vector is any phagemid vector.

14. A process according to claim 13 wherein the display vector is pHEN2.

15. A process according to any of claims 9 to 14 wherein the expression vector is a soluble expression vector.

16. A process according to claim 15 wherein the soluble expression vector is pIM5100.

Patentansprüche

1. Ein Prozess zur Produktion einer antigenspezifischen, antigenbindenden Domäne unter Verwendung eines transformierten Hostes, der eine exprimierbare DNA-Sequenz enthält, welche die antigenspezifischen, antigenbindende Domäne kodiert, wobei sich die antigenspezifische, antigenbindende Domäne von einer variablen Region des Immunglobulin-Isotyp NAR ableitet, der in einer Spezies der Elasmobranchii-Subklasse vorgefunden wird, wobei die Spezifität der antigenspezifischen, antigenbindenden Domäne durch ein Antigen bestimmt wird, das in ein Mitglied der Elasmobranchii-Subklasse eingeführt wird.

2. Ein Prozess gemäß Anspruch 1, wobei der transformierte Host ein Prokaryot oder ein niederer Eukaryot ist.


4. Ein Prozess gemäß eines der vorhergehenden Ansprüche, wobei die exprimierbare DNA-Sequenz die Form eines Phagemidvektors hat.

5. Ein Prozess gemäß eines der vorhergehenden Ansprüche, wobei die Spezies der Elasmobranchii-Subklasse ein Hai oder ein Katzenhai ist.


7. Ein Prozess gemäß eines der vorhergehenden Ansprüche, wobei die antigenspezifische, antigenbindende Domäne auf ein spezifisches Antigen/spezifische Antigene gerichtet ist.

8. Ein Prozess gemäß eines der vorhergehenden Ansprüche, wobei die antigenspezifische, antigenbindende Domäne zu einem einzelnen Antigen erhebt wird.

9. Ein Prozess zur Produktion einer antigenspezifischen, antigenbindenden Domäne bestehend aus den folgenden Schritten:

   a) Immunisierung eines Mitglieds der Elasmobranchii-Subklasse mit einem Antigen;
   b) Isolieren von Lymphozyten von diesem Mitglied;
   c) Isolieren von RNA von den Lymphozyten;
   d) Amplifikation von DNA-Sequenzen, welche die antigenspezifische, antigenbindende Domäne kodieren,
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durch PCR;
e) Klonen der der amplifzierten DNA in einen Displayvektor;
f) Transformation eines Hosts zur Erstellung einer Bibliothek;
g) Auswahl der gewünschten Klonen aus der Bibliothek;
h) isolieren und Purifizieren der antigenspezifischen, antigenbindenden Domäne aus diesen Klonen;
i) Klonen der DNA-Sequenzen, welche die antigenspezifische, antigenbindende Domäne in einen Expressionsvektor kodierte;
j) Transformieren eines Hosts, um die Expression des Expressionsvektors zu ermöglichen.

10. Ein Prozess gemäß Anspruch 9, wobei vor Schritt d) die cDNA der antigenspezifischen, antigenbindenden Domäne erzeugt wird.

11. Ein Prozess gemäß entweder Anspruch 9 oder 10, wobei Restriktionsenzyme verwendet werden, um die amplifzierten DNA-Sequenzen zu digestieren, welche die antigenspezifische, antigenbindende Domäne kodieren.

12. Ein Prozess gemäß Anspruch 11, wobei die Restriktionsenzyme Ncol und NotI sind.

13. Ein Prozess gemäß eines der Ansprüche 9 bis 12, wobei der Displayvektor ein Phagemidvektor ist.


15. Ein Prozess gemäß eines der Ansprüche 9 bis 14, wobei der Expressionsvektor ein löslicher Expressionsvektor ist.

16. Ein Prozess gemäß Anspruch 15, wobei der lösliche Expressionsvektor pMS100 ist.

Revendications

1. Méthode d’élaboration de domaine de liaison d’antigène spécifique de l’antigène consistant à utiliser un hôte transformé renfermant une séquence d’ADN susceptible d’être exprimée et capable de coder le dit domaine, lequel est issu d’une région variable de l’isoforme d’immunoglobine NAR présent dans une espèce de la sous-classe Elasmobranchii et dans laquelle la spécificité dudit domaine est déterminée par l’introduction d’un antigène dans un membre sous-classe Elasmobranchii.

2. Méthode selon la revendication 1 dans laquelle l’hôte transformé est un procaryote ou un eucaryote inférieur.

3. Méthode selon la revendication 2 dans laquelle le procaryote hôte est Escherichia coli.

4. Méthode selon une quelconque des revendications précédentes dans laquelle la séquence d’ADN susceptible d’être exprimée est sous forme de vecteur phagémide.

5. Méthode selon une quelconque des revendications précédentes dans laquelle dans laquelle l’espèce de la sous-classe Elasmobranchii est un requin ou une roussette.

6. Méthode selon la revendication 5 dans laquelle le requin est un requin-nourrice.

7. Méthode selon une quelconque des revendications précédentes dans laquelle le domaine de liaison d’antigène spécifique de l’antigène cible, ou plusieurs, antigène(s).

8. Méthode selon une quelconque des revendications précédentes dans laquelle le domaine de liaison d’antigène spécifique de l’antigène est dirigé contre un antigène particulier.

9. Méthode d’élaboration de domaine de liaison d’antigène spécifique de l’antigène comprenant les étapes suivantes :

a) immunisation d’un membre de la sous-classe Elasmobranchii par un antigène ;
b) isolement des lymphocytes dudit membre ;
c) isolement de l’ARN des lymphocytes ;
d) amplification des séquences d’ADN codant le domaine de liaison d’antigène spécifique de l’antigène par PCR ;
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e) clonage de l'ADN amplifié dans un vecteur d'expression ;
f) transformation d'un hôte visant à élaborer une banque ;
g) sélection des clones recherchés dans la banque ;
h) isolement et purification du domaine de liaison d'antigène spécifique de l'antigène à partir desdits clones ;
i) clonage des séquences ADN codant le domaine de liaison d'antigène spécifique de l'antigène dans un vecteur d'expression ;
j) transformation d'un hôte visant à permettre l'expression du vecteur d'expression.

10. Méthode selon la revendication 9 dans laquelle l'ADNc du domaine de liaison d'antigène spécifique de l'antigène est généré avant l'étape d).

11. Méthode selon les revendications 9 ou 10 consistant à utiliser les enzymes de restriction pour digérer les séquences d'ADN amplifié codant le domaine de liaison d'antigène spécifique de l'antigène.

12. Méthode selon la revendication 11 dans laquelle les enzymes de restriction sont NcoI et NotI.

13. Méthode selon l'une quelconque des revendications 9 à 12 dans laquelle le vecteur d'expression est un vecteur phagémide quelconque.

14. Méthode selon la revendication 13 dans laquelle le vecteur d'expression est pHEN2.

15. Méthode selon l'une quelconque des revendications 9 à 14 dans laquelle le vecteur d'expression est un vecteur d'expression soluble.

16. Méthode selon la revendication 15 dans laquelle le vecteur d'expression soluble est pMS100.
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Figure 2C
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Figure 5B
Figure 6
Figure 7
Figure 10
Figure 12
Figure 13
Figure 14
Figure 15
Figure 16
REFERENCES CITED IN THE DESCRIPTION

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