Exploring antibody engineering technology for diagnostic applications

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Exploring Antibody Engineering Technology for Diagnostic Applications

DE HAARD, Johannes Jozef Wilhelmus

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Johannes Jozef Wilhelmus de Haard

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Promotor :

Prof. Dr. J.W. Arends

Co-promotores :

Dr. ir. H.R.J.M. Hoogenboom

Dr. B. van Gemen (Organon Teknika, Boxtel)

Beoordelingscommissie :

Prof. dr. J. de Haan (voorzitter)

Prof. dr. P.J.C.van Breda Vriesman

Prof. dr. M.P. van Dieijen-Visser

Dr. A.D. Griffiths (Centre for Protein Engineering, MRC, Cambridge, UK)

Prof. dr. P.J. Hudson (CRC for Diagnostic Technologies, CSIRO, Melbourne,
Australia)

Aan Willeke, Monique, Saskia en Eline

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INTRODUCTION

Adapted from

Creating and engineering human antibodies for immunotherapy

Hans J.W. de Haard, Paula Henderikx, Hennie R.J.M. Hoogenboom

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1 Antibodies in *in vitro* diagnostics

In vitro diagnostics find their application in the different disciplines of science, ranging from virology, where they enabled the identification of infected sera, to endocrinology, for the detection of hormones, or in the field of haemostasis. The immuno-assay plays also an important role in fundamental research, where it aids in the characterisation of proteins or other antigens, and certainly it will be applied in the coming era of Genomics for the characterisation of new discovered gene products. Traditionally, antibodies form the basic ingredients of diagnostic products. After the introduction of hybridoma technology [1], when homogeneous batches of monoclonal antibodies became available, the sensitivity and the specificity of the assays improved significantly as the result from the predefined antigen recognition. Substitution of (one of the) polyclonal by monoclonal antibodies for capture or detection reduced background problems, while the use of high affinity antibodies increased the sensitivity of the assays. By their specific epitope recognition, it was feasible to design tests which only detected the desired antigen, and not closely related products.

Genetic engineering of antibodies has already been applied in the field of diagnostics, for instance a commercially available, whole-blood agglutination assay was made with a scFv, directed against the surface antigen glycophorin A of red blood cells, fused to human immunodeficiency virus (HIV) gp36 or gp46 peptides [2,3]. Genetically linkage of the antigen binding domain with desired effector functions might also be useful for immuno-assays: Laukkanen and colleagues expressed immuno-liposomes [4], and *in vivo* produced alkaline phosphatase antibody conjugates have been generated by several groups [5,6]. A new era may have started in the generation of diagnostic reagents by phage display of antibody fragments. This technology has allowed access to large collections of different phage antibodies, created by the cloning of antibody V-genes from B-cells. Antibodies against any type of antigen may be derived from such repertoires, by rounds of enrichment on antigen and re-amplification. Moreover, by the availability of the encoding antibody genes, the selected fragments can be engineered on affinity and the molecular format aiming at their application.

This thesis addresses in detail the engineering and selection of antibodies from display repertoires, aiming at their application in *in vitro* diagnostics. In paragraph 2 of the Introduction, the principles of the repertoire technology are described. These include the expression of antibody fragments in the prokaryotic host *E. coli*, the display on filamentous phage and, associated with this, the issue of the selection methodology, by which phage, that expose antigen specific antibody fragments, are isolated from repertoires. Subsequently the different kind of repertoires and their construction are presented in paragraph 3, while in paragraph 4 we speculate about the perspectives of the technology. Finally, in paragraph 5 the aim of the study will be outlined.

2 Phage display technology

The use of phage to display antibody fragments hinges on the expression of functional antibody fragments in bacterial hosts, which will be reviewed first.

2.1 Expression of antibody fragments in *E. coli*

Correctly folded antibody fragments, Fab, Fv or single-chain Fv (scFv) fragments, are expressed in *E. coli* by routing the nascent antibody chain(s) to the periplasm of the bacterium [7,8] (reviewed in [9]), where the intradomain disulphide bridge between the two β -sheets is formed and the V_H and V_L will pair. Periplasmic expression in the bacterium resembles the natural production route in the endoplasmic reticulum (ER) of the lymphocyte. Both the heavy and light immunoglobulin chains carry a signal peptide, which is cleaved during transport from the cytoplasm over the membrane into the lumen of the ER, where the disulphide bridges are formed. Direct cytoplasmic expression does not yield functional antibody fragments, unless the molecule is refolded in vitro, for example from inclusion bodies [10,11].

Many different antibody formats have been expressed in *E. coli*, including Fab [8,12], Fv [7], scFv [11,13,14], antibodies with one V-gene domain only [15], and fragments thereof [16]. In the scFv, the most popular format, the two variable domains are fused via a flexible linker region, and many hybrid formats. A linker was used to stabilise the Fv-fragment, containing the V_H - and V_L -molecules, because, although spontaneous assembly of V_H and V_L domains occurs in the periplasm of the bacteria, they tend to dissociate upon dilution [14]. The order of the V-domains may be varied, with the V_H -domain at the amino terminus [13] or at the carboxy terminus [11], albeit with adjustment of the linker length for optimal spatial arrangement of the two V-domains [17,18]. The most frequently used format, V_H -(Gly₄Ser)₃- V_L has been used extensively for the construction of phage libraries [19,20,21] (see also further). In a few cases, it has been reported that the V_H -linker- V_L format did not yield a functional scFv, due to involvement of the N- or C-terminal regions of V_H and V_L in antigen binding; for example, the N-terminal V_H region may be oriented closely to the CDR3 of the V_H , and its fusion to a linker sequence may affect antigen binding [22]. In one instance, positive charges in the amino terminal part of the V_H -region were implicated in blocking scFv transport across the bacterial membrane [23]. It should be kept in mind that these factors, apart from technical reasons, may explain the occasional difficulties found in trying to express functional scFv fragments [24,25,26].

Alternatively, Fv may be stabilised by the introduction of a disulphide bridge between the variable light and heavy chain domains [34,27]; when introduced in a scFv, the thermal stability is enhanced also [28]. For some antibodies, such Fv-variants may have better anti-tumor activities than the scFv analogue, for example, when injected as immunotoxin [29,30].

Conversion from the hybridoma-produced antibody into a bacterially expressed fragment might be needed for modelling and humanization [31], for crystallization [32,33,34] or for

analysis of residues in the antibody involved in antigen binding by combining mutagenesis and X-ray diffraction [35]. Since numerous researchers encounter problems when cloning antibody V-genes from monoclonal antibodies for expression in bacterial hosts, it is worthwhile spending some time on its causes. Hybridoma V-genes may be isolated by application of the polymerase chain reaction (PCR) from the B-cell cDNA [36,37,38,39]. Several primer sets for the amplification of murine and rat V-genes have been described (details are given below). The V-genes are then cloned into an appropriate expression vector [40]. Many of the cloning problems are related to not finding the correct, antigen binding V-gene pair [24,25,26,41,42]. This is often caused by the transcription of high amounts of aberrant heavy and light chain mRNAs in the hybridoma, derived from unsuccessful V-gene recombinations in the B-cell or myeloma fusion partner, or the hybridoma is a fusion product of different B-cells. Frequently therefore, a high frequency of non-antigen binding antibody fragments may be found after cloning. Furthermore, the correct V-genes may have acquired mutations associated with the PCR method, or may have lost (part of) the antigen binding due to oligonucleotide-induced changes of the N- or C-terminal region, which unpredictably affect antigen binding (see chapter 1 and 2 of this thesis).

The availability of PCR primers for the generation of large repertoires of V-genes led to the idea of accessing not just one but thousands or millions of different antibody fragments expressed in *E. coli*. From a repertoire of V_H-domains derived from mice immunized with lysozyme, Ward *et al.* [40] isolated anti-lysozyme single domain antibodies, by filter screening the bacteria. Alternatively, phage lambda was used, in combination with plaque screening [37]. Both approaches were restricted to the screening of a limited number of clones, and on the availability of large amounts of labeled antigen. Although they provided the concepts for creating and accessing large antibody repertoires, the real potential of combinatorial repertoires was established using phage antibody display.

2.2 Display of antibody fragments

Antibodies were the first proteins to be displayed successfully on the surface of phage [43]. This was achieved by fusing the coding sequence of the antibody variable (V) regions encoding a single-chain Fv fragment to the amino terminus of the phage minor coat protein pIII. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the antibody being presented on the phage surface, while its genetic material resides within the phage particle. Phage display was originally described in 1985 by Smith [44], who illustrated the use of the filamentous bacteriophage fd for the display of peptides on the phage coat (for review see [45]).

Non-lytic filamentous phage infect strains of *E. coli* that harbour the F conjugative plasmid. The phage particles attach to the tip of the F pilus, encoded by genes on this plasmid, and the phage genome, a circular single-stranded DNA molecule, is translocated into the cytoplasm. The genome is replicated involving both phage- and host-derived proteins, and packaged by the

infected cell into a rod-shaped particle, which is released into the media. All virion proteins will undergo transport to the cell periplasm prior to assembly and extrusion. The particle is essentially the phage genome covered by thousands of copies of a small capsid protein (pVIII), and, at one end of the particle, by five copies of the adsorption protein, the product of gene III, which mediates binding to the F-pilus. For further details on phage biology, see ref. [46].

In 1990, the display of a single-chain antibody fragment on the surface of the filamentous phage was demonstrated, as well as the possibility of selecting the antibody displaying phage from a mixture of irrelevant phage [43]. The first antibody was displayed using a phage vector, based on phage fd-tet [47] and its gene III as fusion partner. In this vector, the genes encoding an antibody scFv fragment are cloned in frame with gene III and downstream of the gene III signal sequence, which normally directs the adsorption protein to the periplasm. Here, the V_H and V_L domains will fold correctly, stabilised by an intramolecular disulphide-bridge, and pair to form a functional scFv. A phage vector carries all of the genetic information required for the phage's life cycle. Phagemids, a more popular vector for display, are plasmid vectors that carry gene III with appropriate cloning sites, and a phage packaging signal (Fig. 1) [48,49,50]. The phagemid

Fig. 1. Display of scFv antibodies on filamentous phage. Bacteria harbouring a phagemid vector, encoding the V_H and V_L immunoglobulin genes fused to gene III of filamentous phage fd, are superinfected with helper phage to drive production of phage particles carrying the scFv, as a fusion product with the phage coat protein pIII, on the surface, and its encoding DNA inside.

DNA encoding the antibody-pIII fusion will be preferentially packaged into phage particles using a helper phage, such as M13KO7 or VCS-M13, which supplies all structural proteins. This includes also wild type gene III, which will compete with the phagemid-encoded gene III fusion for incorporation in the phage particles. In phagemid systems, a promoter is used (for example, derived from lacZ), which may be suppressed (with glucose) to decrease antibody-pIII expression during library amplification. This prevents some of the bias in the population caused by differential toxicity of antibody-pIII fusions. However, the introduction of an additional

transcription terminator abolishes toxicity altogether [51]. For display of the antibody-pIII product, the repressor (glucose) is removed, leading to sufficient fusion product to derive 'monovalent' phage particles [48,50,52]. For display of Fab fragments [48,50,52] the heavy chain fragment (Fd) or light chain ($V_L C_L$) was fused to gene III and the partner chain was co-expressed separately in the periplasm.

Besides gene III, of which there are approximately five copies on each phage particle, antibodies may also be fused to the small major coat protein derived from gene VIII, present at approx. 2,700 copies per wild type phage particle (for review [53]). Display on one of the other minor coat proteins, pVI, as carboxy-terminal fusion, has been reported for cDNA libraries, but not for antibodies. Although gene III protein is involved in infection, the infectivity of antibody-pIII-containing phage particles often appears not to be dramatically affected by the fusion. However, it has been reported that part of the antibody-pIII fusion is proteolytically cleaved at the fusion junction, providing a source of full-length, thus wild-type, pIII [43,54]. Furthermore, besides fusion to full length pIII, fusions to sites downstream of gene III have been reported [24,55,56].

The key feature of the display of ligands on the phage surface is the linkage of phenotype (surface displayed ligand) and ligand genotype, encoded within the phage genome [57]. This allows the enrichment of antigen-specific phage antibodies, using immobilized or labelled antigen. Phage that display a relevant antibody will be retained on a surface coated with antigen, while non-adherent phage will be washed away. Bound phage can be recovered from the surface, reinfected into bacteria and re-grown for further enrichment and, eventually, for binding analysis. This antigen-driven enrichment procedure may be used to isolate even the rarest phage antibody. In this respect, an important feature of the filamentous phage is its resistance to the severe elution conditions that are used for dissociation of ligands: the phage remains infective when treated temporarily with acid, alkali or with denaturing agents such as urea (for review see [57]).

Alternative display methods have been described, but none have matured as much as the filamentous phage system. These include display on other phage hosts (*E. coli* bacteriophage lambda [58,59,60,61], or T4-phage [62]), on prokaryotic hosts or their extensions (flagelli, *E. coli* itself, LamB and OmpA etc.; for review, see [63]), on eukaryotic hosts or their viruses (yeast [64]), including retroviruses [65,66]. None of these systems have been used as extensively as filamentous phage and, to date, neither the construction of large antibody libraries nor the selection of high affinity antibodies has been reported.

2.3 Library technology

The success of antibody phage display hinges on the combination of the display and enrichment method, with the creation of large combinatorial repertoires of phage antibodies, which will be discussed first.

Naturally occurring V-genes may be isolated from B-lymphocytes by application of PCR [37,38,39]. cDNA synthesized from total or poly-A RNA extracted from B-lymphocytes can be used as template for PCR; alternatively the rearranged chromosomal DNA is taken (reviewed in [57]). As a source for genetic material, peripheral blood lymphocytes can be used; or the spleen, lymph nodes, tonsils and bone marrow, with the choice depending on the type of library that will be made.

Fig. 2. Location of primers in antibody genes. Combinations of forward and backward primers that map in the signal, framework or constant regions may be used to amplify antibody V-genes from (un)rearranged DNA or mRNA.

Several sets of "universal" PCR primers, suitable for the amplification of large arrays of V-genes, have been described for repertoire cloning. As is shown in Fig. 2, antibody encoding DNA fragments can be amplified with different combinations of oligonucleotides, based on either the signal sequence, the 5' and 3' end of the variable genes, or on the constant region. The 5' and 3' ends of the V-genes are sufficiently conserved to allow the design of primers for the amplification of mouse variable regions [19,39], or human light and heavy chains [36]. Extensive sets of both family-specific and degenerate primers have now been described, for the construction of human [20,36,67], murine (also usable for rat) [19,24,39,68,69], rabbit [70], or chicken [71,72] V-gene repertoires.

For cloning as scFv- or Fv-fragments, the easiest route involves amplification of the V-genes with oligonucleotides based on the FR1- and FR4-region, and cloning of these fragments into an appropriate display vector. The primers can be designed to contain restriction sites introduced within the antibody V-sequence [40], or may be appended with cloning sites outside the V-region [19]. The choice depends very much on the cloning vector and strategy used. Cloning may be carried out in a stepwise, sequential fashion or may involve a 'splice overlap extension' (SOE [73]) reaction to sow together V_H and V_L genes in scFv or Fab format before

one-step cloning (reviewed in [57]). For the construction of Fab-repertoires, the introduction of isotypes of the C_H1 -gene, which are present in the mRNA, should be avoided, to prevent bias in the repertoire due to expression differences [74], and to simplify the inclusion of the disulphide bond between the heavy and light chain. Provision of this intermolecular disulphide bond is not strictly necessary; the pairing of heavy and light chain is sufficiently stable without this covalent bond. Indeed, Fab repertoires have been made by supplying the same C_H1 domain (often the human gamma-1 C_H1 gene) in the display vector [48,75,76]. Fab repertoires are then constructed by cloning in the V_H gene pool, and the complete light chain (lambda and kappa), again either in a stepwise fashion, or after SOE. An isotype-specific V-gene may be amplified by using appropriately designed isotype-specific oligonucleotides during first strand cDNA synthesis or during V-gene amplification [20].

For cloning, originally, restriction enzymes were chosen that were frequently present in naturally rearranged V-genes, such as *Pst*I in the FR1 of V_H , and *Bst*EII in the FR4 of V_H , the advantage being that, at these positions, the naturally occurring residues were maintained in FR1 and FR4 [39]. In due course, improved designs have appeared which involve the use of restriction enzymes that more rarely cut within antibody genes, on the basis of recognition of 8 bases (*Sfi*I, *Not*I, *Asc*I etc.) [24,77], or on the basis of a study of the frequency of restriction sites in a large database of V-genes [78]. The use of SOE reduces the number of cloning steps, but is a technically demanding procedure, that, if not carried out carefully, may lead to loss of diversity due to excessive use of PCR.

Fig. 3 illustrates the construction of a library. B-lymphocytes, expressing the antibodies, serve as a source of V-genes. After RNA- or DNA-isolation, the V-regions are amplified and randomly assembled to create scFv cassettes. These are supplied by restriction sites at the 5' and 3' ends, and cloned into a phage(mid) vector for expression of fusions of scFv phage coat protein. This procedure will yield random combinatorial libraries (the original pairing of V_H and V_L will be lost).

2.4 Selection strategies

Phage antibody libraries are enriched for antigen-binding clones by subjecting the phage to repetitive rounds of selection, which includes binding, washing and elution steps, re-infection into bacteria and growth/rescue to re-express the antibody on the phage surface. This selection cycle is illustrated in Fig. 4A. (for a review, see [79]). Ideally, only one selection round should be required, but the aspecific binding of phage limits the enrichment that can be achieved per round and in practice two - four rounds are necessary.

In Fig. 4B, the most frequently used in vitro selection methods are described. The first selections of phage antibodies were achieved with affinity columns [19,43] (Fig. 4B, panel a) and later with antigen adsorbed onto plastic surfaces (Fig. 4B, panel b) such as enzyme-linked immunosorbent assay (ELISA) plates or "Immunotubes" [20,52], or antigen immobilised on

BIAcore sensorchips [80]. In all cases, care must be taken to retain the conformational integrity of the antigen, e.g., sometimes antibodies selected against a protein adsorbed onto plastic may not recognise its native form. An indirect coating may be applied, using an antigen-specific

Fig. 3. Construction of a human antibody library displayed on phage. cDNA fragments encoding for the heavy and the light chain variable regions of the antibodies (VH, VL) are amplified from human B-cells by PCR and assembled. The assembled genes are inserted in a phagemid vector in frame with the gene encoding the coat protein pIII. The vector is introduced into *E. coli*. After rescue with helper phage, the random combinatorial library of antibodies is displayed on phage.

antibody for capture [81]; non-specific Ig of the same species and isotype may be added during the selection procedure to avoid isolation of antibodies to this primary reagent. Adaptation of the panning method permits the selection of antibodies with a desired specificity, as with the epitope masking technique [82,83], in which an immuno-dominant epitope was blocked by a mAb. To prevent the selection of cross-reactive antibody fragments, a competitor antigen that is highly homologous to the protein of interest may be added, to provide a competitive deselection [84]. Furthermore, the selection on antigen, which was displayed on the surface of bacteria has been described, which appeared, surprisingly, more efficient than when selecting on purified antigen [85].

These panning or column methods do not easily allow a quantification of the amount of antigen used and, thus, the concentration used during selection. This may be achieved by applying a powerful and, probably, now, the most frequently applied method, based on using labeled soluble antigen such as biotinylated antigen [86]. After incubation of phage mixes with the biotinylated antigen, phage bound to the antigen-biotin complex are retrieved with streptavidin-coated paramagnetic beads and a magnet (Fig. 4B, panel d). One of the advantages of this selection procedure is that the concentration used during the selection can be carefully chosen. During the selection of the phage library, the antigen concentration may be decreased to favour the enrichment of antibodies with a higher affinity [87]. A minor disadvantage of this

method is that antibodies to streptavidin will also be isolated, in particular, when non-immune libraries are used; in cases where a high frequency of streptavidin binders are found, a depletion step with coated streptavidin should be performed.

Fig. 4.(A) Procedures for selection with phage libraries. A phage antibody library is incubated with antigen and non-bound phage are washed away. Specific library phage is eluted and reinfected in *E. coli* for amplification and rescue. Phage particles from this amplified library are used to incubate with antigen in a second round of selection. After two to four rounds of selection, individual (monoclonal) antibodies are analysed.

(B) Selection strategies to obtain specific phage antibodies. Selection of phage libraries by flow through a column with immobilised antigen (a) or by panning on antigen adsorbed onto plastic (b). After washing, specific phage is eluted with acidic or basic solutions; alternatively, elution with an excess of the antigen is possible (c). To avoid conformational changes during coating, selection of specific antibodies to biotinylated antigen in solution is more favoured. Bound and unbound phage antibodies are separated using streptavidin-coated magnetic beads (d). Selection on cells can be done directly by panning on cell monolayers or on cells in suspension; unbound phage are washed away by rinsing the flask or by centrifugation (e). Subtraction via FACS: the cells of interest are fluorescently labeled and separated from the others by cell sorting (f). Tissue- and organ-specific phage antibodies might be obtained by selections on tissue slides (g) or by in vivo selection (h).

Phage antibodies bound to antigen can be eluted in different ways, e.g., with (one step or gradients of) acidic solutions such as HCl or glycine buffers [52,88], with basic solutions like triethylamine [20], with dithiothreitol (DTT) when biotin was linked to antigen by a disulphide bridge [89], by enzymatic cleavage of a protease site engineered between the antibody and gene III [90], or by competition with excess antigen [19] (Fig. 4B, panel c) or antibodies to the antigen [91].

Selections on impure antigens are significantly more difficult, due to the problem of enriching phage antibodies specific for non-relevant antigens. Examples are antigens that cannot easily be purified from contaminants with similar properties, or cell surface receptors that can be functionally retained in lipid bilayers only. Direct panning on cell surfaces may be carried out on adherent cells grown in monolayers, or on intact cells in suspension (Fig. 4B, panel e). This may fortuitously select for antigen-specific phage antibodies, in particular, when using immune libraries in which the frequency of irrelevant phage antibodies will be lower [92,93]. When using panning with a large naïve repertoire [94] on a colorectal cancer cell line, we generated antibodies of all the expected types (Zijlema, Hoogenboom *et al.*, unpublished): (1) antibodies recognising pan-cell markers, (2) antibodies binding to the cancer cell line only and (3) a series of antibodies binding to antigens related to the epithelial cell origin. For the latter antibodies, the exquisite epithelial cell origin of the antigen was confirmed by positive immunohistochemistry on colon tumors derived from cancer patients. For most applications, depletion and/or subtraction methods have to be used to home in on antigen-specific phage antibodies, for example, cell sorting by flow cytometry (Fig. 4B, panel f) [25,95] or magnetic activated cell separation. In this case, the (excess of) non-selected cells serve to absorb irrelevant phage antibodies. A powerful selection was achieved by alternating between different sources of antigen [96], to home in on antigen-specific phage antibodies. To fully understand and appropriately design selection of phage antibodies on cell surfaces, systematic studies to compare the various selection methods [97,98] are required. This will be aided by establishing mathematical models for bio-panning, as was elegantly done for phage displaying peptides [99].

Further selections may be carried out on even more complex mixtures of antigens, for example, on tissue sections (Fig. 4B, panel g; Roovers, Hoogenboom *et al.*, unpublished), or whole tissues [100] which will yield, depending on the type of library used, antibodies to cell surface, intracellular and stromal compartments. This may be of particular interest when targeting rare antigens expressed *in vivo* in certain pathological situations, and accessible on tissue sections only. *In vivo* selections, in which the phage repertoires are injected directly into the blood of animals and certain target organs or tissues are collected (Fig. 4B, panel h), may reveal phage antibodies specific for organ/tissue-specific endothelial cell antigens, as has been demonstrated for peptide phages [101].

Selection from phage antibody libraries provides a new tool for the isolation of novel self antigens, such as tumor- or disease-associated antigens. Both the *de novo* combined V domain

pairs in naïve and synthetic antibody libraries (but particularly the latter) are not shaped by the constraints of the immune system, and have not been skewed against anti-self by in vivo tolerance mechanisms. Therefore, the chances to detect unique self antigens and epitopes are improved dramatically compared with the immunization-dependent hybridoma-based antigen screening. To date, this application has been used for probing lymphocyte- [95] and tumor cell surfaces [92], yielding a number of promising [102], but, as yet, uncloned cell-type-specific antigens. The characterization of the antibodies is hampered by the frequent lack of reactivity in immunostaining [92], the antibody's low affinity [95], or the low abundance of the cell surface antigen.

In general, the obtained enrichment factors during panning vary between 20 and 1000 per round [20,43], but with high affinity antibodies, they may reach 10^5 per round (Roovers and Hoogenboom, unpublished). Depending on the display vector used, the percentage of recovery of a selected clone may vary from 0.01-0.1% to 30% per round. These factors are dependent on the affinity of the antibody, the display efficiency (i.e.. the number of antibody copies per phage), the concentration of the antigen, and the physical method of selection (washing steps, elution method, etc.) [54,79]. In theory, the number of selection steps that can be performed is unlimited, thereby allowing successful selection even with minimal enrichment factors/recoveries. In practice, a minimal efficiency is required, in order not to loose phage antibodies, in particular, when selecting from large libraries (see also below).

The selection procedure will enrich binders from the phage library. To select those phage antibodies with the best affinity, decreasing amounts of antigen should be used, selections should be carried out in solution rather than by avidity-prone panning, and sensitive and accurate affinity screening methods, such as a BIAcore-based affinity screening, should be available.

The enrichment factor is influenced also by the background binding of non-specific phage particles, which necessitates reiteration of the selection procedure. This may be by-passed in some applications by repeating the selection of eluted phage immediately without re-amplification [103]. Two alternative systems have been described, with the aim of reducing the selection step to just one round [104,105]. In both methods, the antibody library is made using non-infective phage particles, by deleting part of the gene III protein. Infection will be restored by binding a fusion protein consisting of the cognate antigen and the two first amino terminal domains of gene III, which are responsible for pilus binding [106]. The fusion product may be supplied *in vitro*; infection of the antibody-displaying phage will be mediated in a small concentration window [107]. In the *in vivo* SIP system (selective infection of phage, [107]), both antibody and antigen are encoded on the same phage genome. Applications of this promising system are hampered by the inefficiency of the restoration of infectivity; even when using an antibody-antigen pair of high affinity, infection using SIP remains at least 10.000-fold less efficient than when using phage carrying the wild-type gene III product. It remains to be seen if these selection systems will be able to replace the more controlled *in vitro* selection methods.

2.5 Screening assays

After the selection procedure, a fast and robust screening assay for binding of poly- or monoclonal phage antibodies is applied, ranging from a simple ELISA with coated antigen, bioassays for screening for direct neutralization upon binding [108], to (for cells) whole cell ELISAs (Lutgerink, Hoogenboom *et al.*, unpublished), Fluorescence-Activated Cell Sorting (FACS) [100] or immunocyto- or histochemistry [109,110,111,112]. For speeding up screening procedures, phagemid vectors that incorporate a dual purpose, obtaining monovalent display of scFv or Fab fragments, and producing soluble antibody fragments for screening [48,56], have been developed. In such systems, an amber codon was positioned between the antibody and pIII gene; in suppressor *E. coli* strains, this allows expression of the fusion product, for incorporation in the phage coat; in non-suppressor strains, this amber serves as a stop codon, yielding soluble antibody fragments. A variety of tags has been described that are appended to the antibody fragment for detection, including the myc-derived tag recognised by antibody 9E10 [20], and the Flag sequence [113]. This set-up will allow the use of unpurified phage antibodies or antibody fragments, present in crude supernatant or periplasmic extracts, for screening assays.

Ideally, the screening assay is linked as closely as possible to the final read-out test for the antibody. Frequently for this, the antibody fragments will need to be purified; fortunately histidine-tags for the popular immobilised metal affinity chromatography (IMAC) [114] are compatible with phage display [115]. For reformatting selected antibodies in alternative formats, fast recloning methods are needed for screening large numbers of clones. An eukaryotic expression vector recently described may be used for one-step recloning of V-genes derived from any phage repertoire, and cloned for expression as Fab or whole antibody, or for targeting to different intracellular compartments [78,116]. Fast read-out systems as well as binding tests are required, in particular for screening methods where the influence of affinity or kinetic behaviour is unclear or less important than the precise epitope that is recognised, e.g. virus or cytokine neutralization assays. For example, methods to quickly test the neutralization of growth factor receptors by Fab cross-linking have been reported [108].

We have recently obtained evidence that ligand-displaying phage themselves may act as receptor agonists (Rousch, Hoogenboom *et al.*, submitted), a feature which will simplify screening for phage-based ligands in search of a(nta)gonistic lead compounds dramatically. But with large libraries at hand, we may go beyond the *in vitro* binding interaction itself, and select for a particular function. Examples of previously demonstrated *functional selection* methods are found in retrieving catalytic antibodies [117,118]; as yet unexplored routes are selections for cell internalization [119], cell survival or killing (induction of apoptosis) upon ligand binding, viral entry, receptor cross-linking and receptor triggering (or its blocking).

3 Antibody repertoire cloning

The choice on which type of phage antibody repertoire to use depends very much on the application and the required affinity of the antibody. Ideally, we would have available universal, antigen-unbiased libraries, from which ultra-high affinity antibodies may be selected directly. Right now, two types of such ‘single-pot’ libraries have been described, the naïve and synthetic antibody libraries (reviewed in [57].) From both types of libraries, antibodies against any chosen antigen may be selected, but the affinities are not always sufficient for the application. The alternative is to start with antigen-biased phage antibody repertoires, using V-genes from immunised B-cells, or to affinity mature the antibodies. We will discuss the differences between these repertoires, summarised in Fig. 5 (see also [79]), starting with the immune repertoire.

Equivalent cell type in the <i>in vitro</i> immune system	Antibody repertoire	Advantages	Disadvantages	Application
Plasma/ memory cells	<ul style="list-style-type: none">• Immune using rearranged V-genes from IgG pool	<ul style="list-style-type: none">• Ag dependent repertoires• Antibodies biased for high affinity• Immunodominant epitopes• Originally controlled for unwanted self	<ul style="list-style-type: none">• Uncontrolled contents• Bias against self• New repertoire for each antigen	<ul style="list-style-type: none">• Analysis of natural immune response• Source of V-genes for chain shuffling• <i>In vivo</i> depletion of unwanted specificities
B cell	<ul style="list-style-type: none">• Naïve using rearranged V-genes from IgM pool	<ul style="list-style-type: none">• One repertoire construction for different antigens	<ul style="list-style-type: none">• Uncontrolled contents• High affinity dependent on library size• Specificity originally biased against self	<ul style="list-style-type: none">• Any specificity with the same library
Pre B cell	<ul style="list-style-type: none">• Synthetic, using cloned, unrearranged V-gene segments	<ul style="list-style-type: none">• Controlled contents• One repertoire construction for different antigens	<ul style="list-style-type: none">• Affinity dependent on library size	<ul style="list-style-type: none">• Any specificity with the same library• Systematic affinity maturation methods

Fig. 5. Comparison between different antibody repertoires (see text for details).

3.1 Immune repertoires

For immune libraries, V-genes are derived from the IgG mRNA of B-cells of an immune source, e.g. immunized animals or, in some instances, human immune B-cells. An immune phage antibody library repertoire will be enriched in antigen-specific antibodies, some of which will also have been affinity matured by the immune system [19,120].

This method gives access to more and sometimes better antibodies than when working with hybridomas. For example, from an immune murine phage antibody library, Chester *et al.* [121] identified an anti-Carcinoembryonic antigen (CEA) antibody with an affinity that was

substantially higher than ever obtained with the hybridoma technology. There are plenty of other examples in the literature of other murine immune phage antibody libraries [96,122,123]. Immune libraries were used to retrieve antibodies to 'difficult' antigens, including native T-cell Receptor (TCR)-V α [124] and specific major histocompatibility complex (MHC)/peptide complexes [96]. Recently, even the derivation of repertoires from rabbit [70] and chicken [71] were reported, both describing the isolation of high affinity antibody fragments.

Disadvantages of this strategy, however, include the time required to immunize animals, the unpredictability of the immune response to the antigen of interest, the lack of immune response to some antigens (self antigens or toxic molecules), and the restriction in generating human antibodies. A major drawback of this method is that, for every new antigen, a new phage antibody library has to be created, which adds one - three months to the procedure. Therefore, when large naive or synthetic antibody libraries are available, the use of immune libraries should be avoided [79].

In the longer term, there may be a few occasions where this route will still be useful, i.e. for the analysis of the humoral immune response in man. Naturally occurring high-affinity human antibodies, for example, those present after viral infection or in patients with auto immune diseases or cancer, may be cloned by phage display, with caution, to aid in identifying the antigen or epitope(s) involved in the immune response. The scrambling of V $_H$ and V $_L$ genes in the process of the combinatorial library construction makes it difficult to discriminate 'original' V $_H$ -V $_L$ combinations from *de novo* formed pairs. The likelihood that original pairs may be retrieved is very low (theoretically, the library size would be at least 10^{12} to retrieve original V $_H$ /V $_L$ pairs from an immune repertoire of 10^6 lymphocytes), although it appears that the heavy chain often dominates the specificity [125]. If it is necessary to maintain the original pair, 'in-cell PCR', in which V $_H$ and V $_L$ genes are assembled within the cell [126] before cloning, may be used, or the B-cells may be enriched on antigen [127,128] and/or cloned by limiting dilution, before amplification of the V-genes.

Phage display has been used extensively to study the characteristics of self-antibodies in auto immune diseases (for recent examples, see [125,129] and references therein). A well studied example of humoral responses in viral diseases involves the immune libraries prepared from the bone marrow of human immunodeficiency virus type 1 (HIV-1) seropositive donors. This library appeared to form a rich source of potent neutralizing antibodies to HIV-1, but also to respiratory syncytial virus (RSV) and herpes simplex viruses HSV-1 and HSV-2 (for review, see [130]). While most high affinity Fabs were selected against the CD4-binding site of the HIV-1 envelope protein gp120, other antibodies directed to different epitopes were isolated using the epitope masking technique or by using peptides [83]. In this thesis, we will also describe the application of immune repertoires, of murine and human origin, for the selection of HIV-1 specific antibody fragments.

In a second application of immune libraries, the immune response itself is used to remove irrelevant antibodies, thus biasing the V-genes before cloning against certain antigens. Animals can be made tolerant to certain irrelevant antigens, after which the relevant antigen as a mixture with irrelevant antigens is used for the immunization. This combination of depletion and enrichment, *in vivo* (tolerization and immunization) and *in vitro* (depletion of phage antibodies before selection on antigen), is extremely powerful, as exemplified by the isolation of human anti-tumor cell antibodies from phage repertoires of cancer patients immunized with autologous tumor cells [92], or from their tumor-draining lymph nodes [131].

3.2 Naive repertoires

The primary immune response involves a large array of IgM antibodies that recognize a variety of antigens. The murine naive repertoire has been estimated to contain $<5 \times 10^8$ different B-lymphocytes, while the human repertoire may be a hundred to a thousand times bigger [57]. This array of antibodies may be cloned as a 'naive' repertoire of rearranged genes, by harvesting the V-genes from the IgM mRNA of B-cells of unimmunized human donors [20], isolated from peripheral blood lymphocytes (PBLs), bone marrow, spleen cells, or from animal sources [132]. The first of such 'single-pot' repertoires [20] was made from the PBLs of two healthy human volunteers and contained 3×10^7 clones. From this pool, antibodies to over 25 different antigens were isolated [20,89,93]. The antigens were either "foreign", such as turkey egg white lysozyme, bovine serum albumin and the hapten 2-phenyloxazol-5-one (phOx) [20], or "self", such as thyroglobulin, Carcinoembryonic antigen, mucin, CD4 and tumor necrosis factor α [89]. When probed with entire red blood cells, scFvs directed against antigens to blood group B, D and E [93] were isolated. The average affinity of these antibodies was similar to the affinity of antibodies seen in the naive primary immune response. This appears to be sufficient to allow reactivity in Western blot, ELISA, FACS analysis, etc. [109]. Recently, much larger scFv repertoires have been made by 'brute force' cloning [94] (using 100 μg of vector ligated to 10 μg of insert transformed into bacteria by several hundred electroporations) yielding 1.4×10^{10} independent antibody clones. As source of lymphocytes PBLs, tonsil B-cells and bone marrow B-cells were taken from 43 non-immunized individuals. From this library, antibodies with affinities typical for a secondary immune response were isolated (average K_a around 10^8 M^{-1}), against all of the antigens tested, with two antibodies, against haptens diethylenetriamine-pentaacetic acid (DPTA) and fluorescein, in the subnanomolar range [94]. For a direct comparison with the smaller sized library from Marks *et al.* [20], the increase in library size by a factor of 200 led to an apparent 100-fold decrease in off-rates for anti-phOx antibodies. Even larger sized naive Fab repertoires may be made by using the *in vivo* recombination system based on Cre/*loxP* recombination system ([133] see below).

By brute force cloning, we generated a huge Fab displayed, human repertoire with 4.1×10^{10} clones, which might be used for the generation of high affinity antibodies for diagnostic

applications. The construction and selection with a number of antigens will be described in this thesis (Chapter 7).

Thus, neither immunization nor affinity maturation are needed for the generation of high affinity (human) antibodies. Major advantages of using very large naïve single-pot repertoires therefore are as follows: (1) one library can be used for all antigens, (2) human antibodies can be isolated, (3) antibodies to self, non-immunogenic or toxic substances can be generated, (4) antibody generation takes two - four rounds of selection (approximately two weeks), and (5) when very large repertoires are available, high-affinity antibodies are isolated directly.

The disadvantages are (1) the lower affinity of the antibodies when smaller-sized repertoires are used, (2) the time and effort it takes to construct these repertoires, in particular, when using the *Cre/LoxP* recombination system (see section 1.3.4) and (3) the largely unknown and uncontrollable contents of these naïve libraries. In particular, the positive and negative selection by the immune system, the potentially limited diversity present in the IgM repertoire, its possible bias due to unequal expression of the different V-gene families and the unknown immune history of the B-cell donor, influence the contents and quality of the library. Therefore, an alternative method, based on the controlled addition of defined and chosen V-gene building blocks to antibody repertoires, was developed, i.e., the synthetic antibody library.

3.3 Synthetic repertoires

An antibody's specificity resides in the six complementarity determining regions (CDRs) that shape the antigen combining site. The two variable domains that compose this site are generated by the immune system by recombination of genetic elements, creating an immense variety of antibodies. There are three sets of genetic elements (V_H -, D- and J_H -regions) for the heavy chain and two sets for the light chain (no D-segment). The V-segment encodes the first two CDRs, the third one is the product of the junction of V/D/J for the heavy chain or V/J for the light chain (for review, see [134]). This knowledge has paved the way for the creation of antibodies entirely outside their natural host. To construct a synthetic antibody library, V-genes are assembled by introducing a predetermined level of randomization of CDRs (or possibly also bordering FR-regions) into, ideally, germline V-gene segments [21]. One of the main advantages over naïve repertoires is that, in such synthetic repertoires, the contents, local variability and overall diversity of the library will be controlled and defined.

The areas of synthetic diversity ought to be chosen to correspond to areas of most natural sequence diversity of the primary antibody repertoire. From structural studies, it has become apparent that five of the six CDR regions (all but the CDR3 of the heavy chain, the most central to the antigen combining site of all CDRs) have limited structural variation, and frequently follow a certain canonical fold [135]. There is no apparent consensus in the heavy chain CDR3 (except for the J-segment-encoded residues at the carboxy-terminus), with an estimated potential diversity of 10^{23} different sequences for this loop only [136]. Thus, the CDR3 of the heavy chain

is the most diverse loop, in composition, length (ranging from 2 to 25 residues) and structure, and this is the CDR region that should be partially or completely randomized using oligonucleotide-directed mutagenesis or PCR-based methods, for constructing synthetic repertoires.

Several synthetic libraries have been designed with the heavy chain CDR3 sequence encoded by oligonucleotide primers encoding a stretch of randomised amino acid residues. In the first synthetic antibody library constructed in Cambridge, UK, a human scFv repertoire was made containing 2×10^7 members, in which 49 cloned germline V_H-genes were provided with a "random" V_H-CDR3 loop with five or eight residues, combined with a fixed light chain [21]. From this repertoire, many antibodies to haptens, and one against a protein antigen, were isolated, delivering the first proof of concept for the "synthetic antibody". Subsequently, the diversity in the CDR3-regions was expanded to cover lengths ranging from four to twelve residues, to match the natural length diversity of this loop better [109], and eventually 47 light chain segments with partially randomized CDR3 were added [76]. In the final repertoire, nearly 100 cloned, human V-gene segments were used, covering over 95% of the segments used in the natural human repertoire and, therefore, resembling the human primary antibody library in the diversity of all but the heavy chain CDR3 [71]. The repertoire, with 6.5×10^{10} members was made by *in vivo* recombination (see below), and contained hapten- or protein-specific Fabs with nanomolar affinities, typical for antibodies from a secondary immune response.

Several other synthetic or semi-synthetic repertoires have been made, varying from the most simple approach being to use one rearranged V-gene pair [137], using a defined set of V-gene segments (derived from the first synthetic repertoire [21]) extended with six light chain segments [138], to using an undefined mix of human germline V-gene segments, with randomised CDR3s [139], to diversifying all three CDR loops in one V-gene segment [140,141]. In the first approach, only the full CDR3 region (with sixteen amino acids) of the heavy chain was randomised, limiting the diversity of the repertoire in all other loops and creating a library resembling a constrained peptide library positioned on an antibody scaffold [137]. In the last example of the series, it may be expected that randomising the CDR loops other than the CDR3 of the heavy chain, without attempts to retain the residues involved in the canonical fold of these CDRs, may lead to a substantial fraction of the phage antibodies being misfolded, if expressed at all. Although some antigen-specific antibodies can still be selected from such libraries [140], they may run the risk of being much more immunogenic than antibodies with more naturally appearing CDR regions.

Clearly, many different synthetic library concepts will work to create antibodies of intermediate affinity. However, due to limitations in transformation efficiency, the total theoretically designed diversity normally exceeds practically accessible diversity. For example, when using mutagenic oligonucleotides to randomize six codons (with at each triplet, the first two base positions A, G, C or T, and the last G or C), one billion combinations arise. It is

therefore better to limit the diversity to one or two CDR areas in the primary repertoire, ideally the heavy and light chain CDR3s, since these are naturally the most diverse sequence. After selection, the other CDR loops may be targeted with diversity, while conserving within these CDR regions the canonical positions governing their main-chain conformation. The next section will deal with how larger repertoires may be made, such that a larger number of antibody variants (10^{12}) may be screened for activity.

3.4 Making large repertoires

Typical library sizes made by direct cloning of antibody genes as PCR products are around 10^6 - 10^8 . For these libraries, the total number of different V-genes outnumbers the number of independent colonies that is obtained by direct transformation of *E. coli*. It can be argued on theoretical grounds that, the larger the immune antibody repertoire is, the higher the chance will be to retrieve an antibody with a certain threshold affinity for a given epitope [142], and the higher the chance of finding antibodies with higher affinity. Indeed, from naive or synthetic repertoires with much larger total library size (10^9 - 10^{10} clones), antibodies with affinities typical of the secondary immune response were directly isolated [76,94]. This section reviews how to create large size repertoires.

Besides 'brute force' cloning (see section 1.3.2), repertoires may be made larger than average by cloning the antibody genes as purified restriction fragments (Hoogenboom, unpublished). This procedure involves the separate cloning of V_H and V_L gene pools that had undergone PCR, followed by a second cloning step in which one of the repertoires is cloned as a digested restriction fragment into the phagemid containing the other repertoire (alternatively SOE may be applied, provided that very long (>100 bases) overhangs are provided to allow efficient cutting of the assembled V-genes). Due to the increase in efficiency of ligation with well cut DNA fragments, this method has generated libraries with 10^9 - 10^{11} members (de Haard and Hoogenboom, unpublished).

The ceiling of the size of phage displayed repertoires can be more dramatically extended by applying a combination of the dual antibody cloning strategy [48] and a smart *in vivo* recombination system [133]. This involves the separate cloning of heavy and light chain repertoires: the light chain repertoire is cloned into a phage vector carrying two short recognition sites (*loxP*) for Cre recombinase, one of which is in frame with gene III, while the heavy chain Fd repertoire is cloned into a plasmid bordered by the same two *loxP* sites [133]. By using a wild type and a mutant *loxP* site that cannot recombine, the heavy chain will not be recombined out, but may be targeted to recombine with the identical sites based on the phage genome. After infection of bacteria carrying these plasmids with the light chain-containing phage, the bacteria are superinfected with bacteriophage P1, to produce Cre recombinase. This enzyme mediates recombination between the identical *loxP* sites and, thus, the transfer of the heavy chain to the phage genome. The progeny is a repertoire of Fabs on phage, the heavy chain of which is fused

to pHII. The diversity is now limited only by the number of bacteria that can be infected, thus, 10^{12} in shaker flasks, but 10^{14} when using fermentors (providing that the combined potential initial heavy and light chain diversity is larger, i.e. 10^6 for V_L and 10^9 for V_H). From a synthetic antibody library assembled from nearly 100 V-gene segments, with randomized CDR3s, with over 60 billion clones, indeed antibodies with nanomolar affinities were isolated [76]. Increasing the library size thus results in the isolation of higher affinity antibody fragments, with affinities typical for in vivo matured antibodies, in spite of the absence of somatic mutations in the CDR1 and CDR2 regions of these antibodies.

Besides for antibody repertoires, this in vivo recombination strategy was successfully used for the assembly of a very large peptide repertoire ($>10^{11}$ members) [143]. Two separately cloned 10-mer peptide repertoires were combined, in a similar way to the heavy and light chain repertoires, via in vivo recombination, onto one genome. In this instance, a trick had to be found to create one linear peptide library comprising combinatorial variants of the two starting libraries. For this, the *loxP* site was introduced into a ribosomal self-splicing intron, from *Tetrahymena thermophila*, which, after recombination, remained the only sequence separating the two 10-mer libraries. In vivo splicing at the RNA level then removed most of this intron, leaving only five 'residual' amino acids, separating the two randomised regions [143]. Such clever systems may also be used for creating large scFv (or, with appropriate design, other protein) repertoires, in which the intron is placed in between V_H and V_L .

The larger the library is, the higher the chance will be of retrieving higher affinity phage antibodies from the library, in theory [142]. However, there are physical limitations on the enrichment that may be achieved in one round of selection, which limits the size of accessible genetic diversity. This is illustrated by an example. After selecting on antigen with 10^{13} infectious phage particles from a library with over 10^9 different antibody variants, typically approx. 10^6 phage clones are recovered. This indicates that an enrichment of 1000-fold (or recovery of 0.1%) is required to select the one rare antigen-specific antibody in this population. If the enrichment factor is lower (which will be the case for many of the antibodies), there is a substantial chance that the clone is lost. Thus, with enrichment factors never reported to be higher than 10^5 per round, and with 10^6 clones eluted in the selection, the total genetic diversity accessed is at most 10^{11} clones. If selection conditions are so stringent that very few phage particles are recovered in the first round (typically for, for example, cell sorting via FACS), chances are that different subsets of antibodies will be selected every time this first round is repeated. It, therefore, appears that accessing even larger libraries will not generate even better antibodies. It becomes instead more crucial to optimise the quality of the displayed antibodies (with regard to display levels), and the selection procedures itself.

The construction of even larger libraries may be possible, one day, using a system that bypasses in vivo amplification and phage-background. This is based on the use of *in vitro* polysomes, in which mRNAs are transcribed *in vitro* from short DNA sequences and translated

in cell free systems [144]. The polysome complex containing the encoding mRNA and are translated amino acid sequence are utilized for selection with a ligand. The mRNA from selected polysomes is converted into cDNA and used for the next transcription, translation and selection round. The fact that antibodies may also be *in vitro* translated to form functional molecules, reported in several cell-free translation systems [145,146], is promising; however, it remains to be seen whether these *in vitro* methods can compete with the rather robust *in vivo* display technologies.

4 Perspectives

4.1 Perspectives on selection technologies

The use of *E. coli* as the expression host and filamentous phage as the display host have made antibody engineering fast and relatively simple. The use of very large naïve or synthetic libraries will yield antibodies with similar affinities to the ones generated with immune libraries, but much faster. For example, the affinities of the best antibodies directed to the hapten pHx, selected from an immune repertoire [19], were of the same range as those isolated from a very large synthetic repertoire [76]. Thus, the availability of pre-made large collections of non-immune repertoires has superseded the use of immune repertoires [79]. It should be carefully considered if immunization and the creation of animal-derived antibody libraries is required, and also whether or not such experiments can be justified from an ethical point of view.

Overall, the impression now may be that slightly better antibodies are generated from naïve [89] than from synthetic repertoires [76], however, the advantages in using synthetic antibody libraries [79] will eventually reduce primary naïve libraries to useful but, in time, transient, sources of V-genes. A drawback of these methods is that the construction of libraries is technically difficult, but it is not unlikely that within a few years very large repertoires will be commercially available, like many other cDNA and genomic libraries, and peptide phage libraries. If the selection efficiency of phage libraries may be improved, for example, by using the infection-dependent methods such as SIP, multiple rounds of panning and replication may be avoided, and such process may even lend itself to being automated. Perhaps eventually the technology will lead to the development of a "panning-machine", with which every researcher will select a set of high affinity monoclonal antibodies against his or her favourite antigen or epitope, within the course of a few days.

The use of phage antibody libraries as a drug discovery tool has just begun. Interesting new methods for guided selection from crude mixtures, including "pathfinder" (Osbourne, Johnson *et al.*, pers. comm.), will extend the scope of application of these libraries tremendously. Combining the design and generation of millions to billions of different ligands, with a function-based selection procedure rather than selection for binding, will open even more challenging

applications of this inspiring technology, and provide us with a new handle for drug discovery well into the next decade.

4.2 Perspectives on antibody engineering

By using phage display, we can generate completely human antibodies, and the provision of the V-genes facilitates further engineering of the antibody's characteristics. With this technology, antibody engineering for the first time may be used to design antibodies from scratch, with an option to choose their building blocks, affinity (up to the picomolar range), format (size and valency) and effector function (natural (IgG) or novel (enzymes etc.)). With these antibodies and derivatives, the role of affinity and dissociation rate, size and valency might determine which molecular format will be applied in a certain *in vitro* diagnostic. Tailor-made reagents, therefore, will be generated for *in vitro* but also for *in vivo* diagnosis and finally for therapy.

5 Aim of the study

For the development of *in vitro* diagnostics, monoclonal antibodies play an important role. Antibody repertoire technology seems to be an attractive alternative for hybridoma technology, since it allows the rapid isolation of monoclonal antibody fragments, and, as a consequence of the availability of the encoding antibody genes, the subsequent engineering of affinity, avidity and fusion to reporter enzymes. This thesis aims at the evaluation of repertoire technology for the generation of antibody fragments, useful as diagnostic reagents. The major targets in terms of the product range of the diagnostic company Organon Teknika were chosen, since it enables a direct comparison on epitope recognition and affinity with traditional monoclonal antibodies, that are used in products.

Antibody engineering was set up in at Organon Teknika after the first reports demonstrating the functional expression of antibody fragments in *E. coli* [7,8], but especially the publication of Ward and colleagues [40] in 1989, in which the idea of repertoire cloning was launched, gave the inspiration to start. As many others did in those days, a hybridoma derived monoclonal antibody (directed against the hormone human chorionic gonadotropin (hCG)) was used as a model, since combining only one heavy chain with one light chain variable region fragment would always yield a functional scFv. Unfortunately it did not, and even increasing the number of monoclonal antibodies for the conversion in bacterially expressed fragments did not improve the outcome. The problems were caused by a mutation in framework 1 of a certain family of heavy chains, which was introduced by the oligonucleotide primers used for amplification of the V-regions. Chapter 1 summarises the identification of the mutation by application of engineering technology, and the analysis of the effect on folding of the antibody. Chapter 2 describes a primer induced mutation within FR1 of a certain light chain family, which does not affect the

overall folding found for the heavy chain mutation, but probably dispositions the CDR1 of V_L and thereby affects antigen binding. For the quantification of the hCG-scFv's, having the complete wild type sequence or with introduced mutations, we developed a method based on surface plasmon resonance, that is described separately in Chapter 3.

The solution for the non-functional, monoclonal antibody derived scFv's, was given in 1990 by the display of antibody fragments on filamentous phage, described by McCafferty and colleagues [43]. Using the hCG mABs as starting point small repertoires were constructed, with sets of primers for amplification of the V-regions, from which clones expressing functional scFv's were selected by biopanning on hCG, and from which the destructive mutations could be deduced. At that time, only two years after the introduction of immune repertoires in 1991 [19], it was just a small step to by-pass hybridoma technology and use repertoire cloning for the generation of monoclonal antibodies. In Chapter 4 anti-hCG antibodies, obtained by selection from a murine combinatorial library, were evaluated. The specificity as well as the affinity were studied in detail, and both characteristics were compared with those of monoclonal antibodies, which find their application in diagnostic assays. Both aspects are crucial for the development of a sensitive and rapid, hCG specific immuno-assay.

Repertoire technology was further applied in the context of HIV-1 detection. Chapter 5 describes the isolation and characterisation of anti-capsid protein (p24) scFv's from a library, made from an immunised mouse. Based on the article, published in 1991 by Marks and colleagues [20], it was decided to construct a human immune repertoire using the lymphocytes from an HIV-infected individual, thereby superseding the immunisation of mice. The selection and characterisation of the obtained anti-envelope antibodies are discussed in Chapter 6.

Finally, the application of naive repertoires, already described in 1991 [20], but improved significantly in 1996 [94], eventually avoids immunisation and the repeated construction of immune repertoires. In 1997 a huge human naïve repertoire was generated using Fab as the displayed fragment, and a large panel of antibodies was selected against the hapten 2-phenyloxazol-5-one (phOx) and a number of protein antigens. Chapter 7 describes the construction and the successful selection of antibodies with unique specificities against the diagnostically relevant glycoprotein hormones hCG, hLH and hFSH from this repertoire.

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CHAPTER 1

EFFECT OF A FR1 MUTATION ON FOLDING OF VH

Published as

Absolute Conservation of Residue number 6 of Immunoglobulin Heavy
Chain Variable Regions of class IIA is required for correct folding

*Hans J.W. de Haard, Bert Kazemier, Arie van der Bent, Peter Oudshoorn,
Piet Boender, Bob van Gemen, Jan-Willem Arends and
Hennie R. Hoogenboom*

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1. Abstract

While studying the expression of single-chain antibodies (scFv) derived from several murine monoclonal antibodies, we found that residue 6 in Framework region 1 of the heavy chain variable domain plays a crucial role in antibody folding. Binding activity of three murine antibodies with a heavy chain variable region (V_H) subgroup IIA was completely lost when at this position the wild type residue glutamine (Q) was substituted by glutamate (E). Increased sensitivity towards trypsin digestion of soluble scFv suggested that the lack of binding activity was caused by incorrect folding of Q6E mutants. Grafting of the three additional class IA derived FR1 residues, based upon the comparison between both classes of V_H-sequences, onto the "defect" subgroup IIA sequence, restored antigen binding activity of the Q6E-containing scFv partially. Our results suggest that residue 6 of the heavy chain may be part of a folding nucleus, involving the first two β strands of Framework region 1. The evolutionary conservation of either glutamine or glutamate at position 6 in different antibody families may well indicate, that within immunoglobulin V_H domains, different family specific folding nuclei have evolved.

2. Introduction

The antigen combining site of an antibody consists of the heavy and light chain variable domain (V_H and V_L). Each domain contains four framework regions (FR) and three complementarity determining regions (CDR), based on the region's variability in sequence. The 'conserved' framework regions are involved in the formation of two antiparallel β -sheets typical for the "immunoglobulin fold". The 'hypervariable' CDRs form loops pointing out of the β -strands and are frequently involved in contacting the antigen.

Although occasionally found to be involved in a direct interaction with antigen (Sheriff *et al.*, 1987; Padlan *et al.*, 1989; Bentley *et al.*, 1990), most FR residues play a passive role, taking part in the β -sheet scaffold upon which the CDRs are imposed. The numerous studies on CDR-grafted antibodies (Verhoeyen *et al.*, 1989; Riechmann *et al.*, 1988) have provided a clearer insight on the important and sometimes dramatic influence of FR-residues on antigen binding. While grafting CDRs derived from a murine antibody onto a human FR-scaffold to transplant its specificity, it was found frequently necessary to graft murine FR-residues to re-establish the affinity of the original murine antibody (Riechmann *et al.*, 1988). These FR-residues may play an important role in maintaining the structure of one or more of the CDRs (Chothia *et al.*, 1992; Tramontano *et al.*, 1990); indeed, the supportive role of the FR residues located at the edges of the CDRs (dubbed the 'Vernier'-residues) are particularly well studied (Foote and Winter, 1992). The role of the FR residues in determining the affinity of the antibody was recently underlined

by selection of a number of anti-lysozyme antibody D1.3 FR-residue variants with improved affinity over wild-type (Hawkins *et al.*, 1993).

FR regions, and particular those in β -turns, have also been found to influence *in vivo* folding of antibody fragments when produced in bacteria. There are two principle factors that limit expression in bacteria, the first one being related to the folding of the antibody fragment in the periplasmic space, and the other related to the stress imposed by the antibody on the bacteria. The folding efficiency of the antibody fragment may depend on a single amino acid, which determines the kinetic partitioning between folding and aggregation (Knappik and Plückthun, 1995). Some residues may yield folding intermediates with a higher propensity to aggregate, reducing expression yields *in vivo*; the effect of point mutations on antibody expression therefore is mostly unpredictable.

In this report the unexpectedly drastic effect of mutations in FR1 residues of V_H on the folding of single-chain Fv fragments in bacteria is described. During the conversion of three different mouse antibodies recognizing the human chorionic gonadotropin (hCG) (Birken *et al.*, 1990) into bacterially expressed scFv antibodies, variable effects on antigen binding were noted when analyzing variants with mutations in the FR1 area of V_H class IIA containing antibodies. Both heavy chain mutants with wild-type residue Q replaced by E at position 6 showed complete loss of binding, which was restored by introducing other mutations in FR1. We would like to speculate that this FR1 residue may be involved in a family-specific folding nucleus in antibody V_H domains.

3. Materials and methods

3.1 Antibodies and vectors

Three murine monoclonal antibodies (IgG1, kappa) directed against hCG were used for the conversion into scFvs. These are coded AB2, AB4 and AB3; the first two are reactive against β -core fragment, the latter against β -CTP (nomenclature see Birken *et al.*, 1990). RNA was isolated (Chirgwin *et al.*, 1979) from 5×10^7 hybridoma cells yielding 600 μ g RNA. 0.5 μ g total RNA was transcribed into random primed cDNA, which was used as template for the polymerase chain reaction (PCR).

Phagemid vector pVI, identical to pHENI (Hoogenboom *et al.*, 1991) except having a FLAG-TAG (Hopp *et al.*, 1988), was a gift from P. van Weezenbeek (Organon Int., Oss, The Netherlands). It was constructed in two steps: by PCR assembly (Jayaraman *et al.*, 1989) of two oligonucleotides encoding a new Shine Delgarno-sequence, the pelB-leader and the FLAG-epitope, and subsequent coupling of the obtained fragment to a DNA-fragment coding for geneIII (amplified from phage fd-tet (16)) by splicing by overlap extension polymerase chain

reaction (SOE-PCR) (Horton *et al.*, 1989). The resulting fragment was digested with *EcoRI* and *HindIII* and cloned into phagemid vector pSSL1190 (Pharmacia, Lund, Sweden).

The "T"-vector was prepared from pGEM7zf(+) (Promega, Madison, USA) as was described before (Marchuk *et al.*, 1990).

3.2 Cloning antibody genes

For cloning antibody V-regions we have originally used oligonucleotides with restriction sites located within the variable regions, similarly to Orlandi *et al.*, 1989 (primer set 1 in Table I). Amplified DNA was gel purified using the QIAEX system (Qiagen, Hilden, Germany) prior to digestions. V_H and V_K encoding PCR fragments were cloned sequentially in pVI using *PstI* or *XhoI* and *NcoI* for V_H and *SalI* and *BclI* for V_K. Electroporations (Dower *et al.*, 1988) were performed in either JM101, a suppressor strain used for phage display when using amber-containing phagemids (Hoogenboom *et al.*, 1991) or TOP F', a non suppressor for expression of soluble scFv.

For the determination of the complete wild-type regions V-genes were amplified on random primed cDNA with primers located in the constant region and in the leader sequence (primer set 2 Table I). The PCR-fragments were cloned into the T-vector and completely sequenced (sequences are shown in Fig. 1).

To amplify V-genes for the construction of phage libraries, we designed primers with restriction sites *SfiI* and *NotI* located outside the variable coding region as in Clackson *et al.*, 1991 (primer set 3 Table I). DNA fragments encoding V_H, V_K and the linker were gel purified and combined in a SOE-PCR for assembling the entire scFv encoding gene segment.

The scFv-wt constructs were made in a similar way by amplification with primer set 4 (Table I).

Mutants were constructed by application of SOE PCR with primers encoding the mutation and having an introduced diagnostic (silent) restriction site; the number of cycles was limited to 20. In order to exclude PCR-mistakes the inserts of all clones were completely sequenced with M13 rev-primer, the geneIII-primer, linkfwd and linkrev (see Table I).

3.3 Panning of phage libraries

Rescue of phagemid particles, selection, washing and elution was performed as described (Marks *et al.*, 1991). Six well tissue culture dishes (Costar, Cambridge MA., USA) were coated with highly purified hCG (Canfield and Morgan *et al.*, 1973) at 3 µg/ml in 50 mM NaHCO₃ pH9.6 and used for selections.

A. Heavy chain

Antibody	Subgroup	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
		10	20	30	40	50	60	70
#2	IIA	QVQLSQQSGFPELVKPGASVMECKASQYFTT	DYVLS	WYKQRTQQLKMTG	ELYPGSHNTYHNEKFD	KATLTADSHNTAYMQLSLTSHDAVYFCAR	MDVYGGSHMAYFN	MGASTVTYVES
#3	IIA	E--Q--TV--AR--I--D--A	S--NH	--P--	A--NHSA--H--V	--K--VT--AT--I--T--N--Y--T	GGDYKEDL	VY--Q--L--
#4	IIA	Q--Q--T--AS--TL--	--A--	--T--	GI--F--R--G	--M--K--	PF--DYDKL	AY--Q--L--A
MBR1	IIA	E--QE--T--AS--TL--	--HI--N	--K--P--	--V--G--Q--MG	--F--V--R--V--V--N--Y--G		
TFS-139	IIA	E--QE--T--AS--TL--	--HI--N	--K--P--	--V--G--Q--MG	--F--V--R--V--V--N--Y--G		
MOPC-104E	IIA	E--QE--T--AS--TL--	--HI--N	--K--P--	--V--G--Q--MG	--F--V--R--V--V--N--Y--G		
IBAF	IIA	E--QE--T--AS--TL--	--HI--N	--K--P--	--V--G--Q--MG	--F--V--R--V--V--N--Y--G		

B. Light chain

Antibody	Subgroup	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
		10	20	30	40	50	60	70
#2	IV	KIVLTQSPAIHSASLGSRVHTTC	TASSS	VSSSYLH	WYQKTSQSPFLMIY	STNKLAS	GYPARFSGSGSGTSTSLTSSMEADATYYC	HQVHRSP YA
#3	II	DV--R--T--LRL--VTI--QASIS--	RS--QLLYG	WRT--H	--L--R--Q--RL--	LV--K--D--	--D--T--DFT--E--RV--LGL--	W--GTH--HT
#4	II	DV--R--T--LRL--VTI--QASIS--	RS--QLLYG	WRT--H	--L--R--Q--RL--	LV--K--D--	--D--T--DFT--E--RV--LGL--	W--GTH--HT
IBAF	IV	Q--Q--T--AS--TL--	--A--	--T--	GI--F--R--G	--M--K--	PF--DYDKL	AY--Q--L--A

Fig. 1. Deduced amino acid sequences of the variable heavy (A) and light (B) chain regions of anti hCG monoclonal antibodies AB2 and AB4. Numbering of the residues and classification of the heavy and light chains was performed according to Kabat (Kabat *et al.*, 1991). In addition the heavy chain V-regions are shown of MBR1 (a class IIA member with glutamate on position 6), TFS-139 (class IA) and MOPC104-E (class IIA). On the bottom lines are the V_H and the V_L sequences listed of the anti 2,2,6,6-tetramethyl-1-piperidinyloxy-dinitrophenyl antibody coded IBAF (Leahy *et al.*, 1988).

Table I. Oligonucleotide primers used for amplification and cloning of variable regions.

primerset	primer name	sequence	site
#1	VH-FR1	5'-AG(G/A) TGC A(G/A)C TG C TCG AG T C(T/G)G G	Xho I
	VH-FR1	5'-AGG T(C/G)(A/C) A(A/G) C TCG AG (C/G)	Pst I
	VH-FR4	5'-TGA GGA GAC GGT GA C CAT GG T CCC TTG GCC	Nco I
	VK-FR1	5'-GAC ATT CA G TCG AC C CAG TCT CCA	Sac I
#2	VH-FR4	5'-GTT TGA TCA CCA GCT TGG TCC C	Bcl I
	VH-leader	5'-AGC TGG ATC (T/C)TT CTC TT	
	CH1-IgG1	5'-CAG ACC CAG GGG CCA GTG	
	VK-leader(AB2)	5'-ATC AGT GTC ACA GTC ATA (A/G)T	
#3	VK-leader(AB4)	5'-T(T/G)G TG(C/T) TCT GGA T(C/T)C CTG	
	CK	5'-TGG ATG GTG GGA AGA TGG AT	
	VH-FR1	5'-aat a gg ccc agc ccc agc cgg gttg cc(G/C) AG(G/A) TCC AGC TG(C/G) AGC AGT CTG G	Sfi I
	VH-FR4	5'-ccT G(A/C)G GAG ACG GTG A(C/G)C (A/G)(T/G)A GTC CC	
#4	VK-FR1	5'-gga tct (A/G)AA (A/G)TT GTG (C/A)TG ACC CAG (T/A)CT C	
	VK-FR4	5'-tat acc g gc ggc cgc CCG TTT GAT CAC CAG CTT	Nci I
	VHLINK	5'-GGT CAC CGT CTC CTC Agg	
	VKLINK	5'-GAG (A/T)CT GGG TCA (G/T)CA CAA (T/C)TT (T/C)ag atc c	
#5	VH-FR1 AB2	5'-aat a gg ccc agc ccc agc cgg gttg ccC AGG TCC AGC TGG AGC TGG AGC AGT CTG G	Sfi I
	VH-FR4 AB2	5'-ccT GAG GAG ACG GTG ACC GTG GTC CC	
	VK-FR1 AB2	5'-gga tct AAA ATT GTT CTC ACC CAG TCT CCA	
	VK-FR4 AB2/4	5'-tat acc g gc ggc cgc CCG TTT TAT TTC CAG CTT GGT (C/G)CC	Nci I
#6	VHLINK AB2/4	5'-GGT CAC CGT CTC CTC Agg	
	VKLINK AB2	5'-AGA CTG GGT GAG AAC AAT TTT aga tcc	
	VH-FR1 AB4	5'-aat a gg ccc agc ccc agc cgg gttg ccC AGG TTC AGT TGC AGC AGT CTG G	Sfi I
	VH-FR4 AB4	5'-ccT GCA GAG ACA GTG ACC AGA GTC CC	
#7	VK-FR1 AB4	5'-gga tct GAT GTT GTG ATG ACC CAA ACT CCA	
	VKLINK AB4	5'-AGT TTG GGT CAT CAC AAC ATC aga tcc	
sequencing	genelIII	5'-tga att ttc tgt atg agg	
	linkfwd	5'-ctc ggg cgg tgg tgg gtc	
	linkrev	5'-gac cca cca ccc gag	

3.4 Refolding of scFvs

The cells from a 50 ml culture were applied for refolding by resuspending in 8 ml 8 M urea solution (in PBS). After sonication the mixture was rotated head over head for 30 min and insoluble material was removed (centrifugation for 30 min at 13000xg). The supernatant was dialysed against PBS with 4 buffer changes. Insoluble protein was removed by centrifugation (30 min at 13000xg) and the supernatant was passed through a 0.2 µm filter.

3.5 Characterisation of scFvs

Large scale productions were performed as described before (Marks *et al.*, 1992) in 50 ml medium. After induction with IPTG growth was continued for 4 h at 30°C. Cells were pelleted and used for the preparation of periplasmic fraction using the borate buffered saline (BBS) shock procedure (Skerra and Plückthun, 1991).

Production of individual clones was monitored on 12% polyacrylamide gels, which were blotted on nitrocellulose. ScFv was detected with anti-FLAG M2 (Kodak IBI, New Haven, USA) 1/4000 diluted in PBST, as conjugate we used anti-mouse peroxidase (DAKO, Glostrup, Denmark) 1/4000 diluted in PBS. The conjugate was detected with the ECL-detection kit (Amersham, Buckinghamshire, UK).

For ELISA microtiterplates were coated with 4 or 10 µg/ml purified hCG in 50 mM NaHCO₃ pH 9.6 (overnight at roomtemperature). The wells were blocked with BSA (0.2% (w/v) in 0.1 M Tris pH 7.4, 30 mM KI). The periplasmic fractions or refolded scFvs were diluted in sample dilution buffer (PBS containing 20% (v/v) normal goat serum and 1% Triton X-100). Bound scFv was detected with anti-FLAG and anti mouse peroxidase conjugate. Staining was performed with tetramethylbenzidine (TMB) and ureaperoxide as substrate.

The amount of scFv was determined on Western blot with a model GS-700 Imaging Densitometer (BIORAD, Hercules, CA, USA) using a standard curve of purified scFv.

For kinetic analysis on the BIAcore, CM-chips (Pharmacia, Lund, Sweden) were applied for covalent coupling of hCG or anti FLAG antibody M2. Experiments were performed with a high density coat of hCG (4369 response units (RU)) or anti-FLAG (9567 RU) (see Figure 3).

3.6 Trypsin digestion

The periplasmic fractions prepared from clones AB2-WT and AB4-Q6E were combined (containing approx. 50 ng of each scFv) in a single digestion reaction containing 100 mM Tris-HCl pH8.0 and 10 mM MgCl₂ and 200 or 40 µg/ml 2.5% trypsin (ICN Biomedicals Inc, Costa Mesa, CA, USA). Alternatively, a mixture was made of the extracts from clones AB4-WT and AB2-Q6E. After 2 hours incubation at 16°C the digestion was stopped by adding Laemmli buffer. The samples were analysed on Western blot.

4. Results

4.1 *V_H*-FR1 mutations affecting antigen binding

Two different murine anti-hCG monoclonal antibodies, binding different regions on the β core of hCG and coded AB2 and AB4, were converted into single-chain Fv's (scFvs) and expressed by secretion in bacteria. Initially the scFv cassettes were amplified using oligonucleotides with restriction sites located within FR1 and FR4 (as in Ward *et al.*, 1989), but the resulting clones, AB2/4-Pst and AB2/4-Xho in Table II, were surprisingly inactive. In an alternative approach small phage display libraries were made, using degenerate oligonucleotide primers with cloning sites based outside the variable regions (as in Clackson *et al.*, 1991). After selection on hCG, several functional scFvs were found (AB2/4-PH1 and AB2/4-PH2 in Table

Table II. Antigen binding characteristics and FR1 sequences of the recombinant scFvs.

AB	Clone	sequence ^a FR1-VH	Prod ^b $\mu\text{g/L}$	ELISA ^c OD ₄₅₀	$\mu\text{g/L}$	BIA ^d RU	ahCG $\mu\text{g/ml}$
AB2		12345678					
	WT	QVQLQSG	355 $\pm$ 42	0.37 $\pm$ 0.04	178	320	1.8
	PH1	E---Q---	618 $\pm$ 17	1.79 $\pm$ 0.03	309	695	3.1
	PH2	-----	630 $\pm$ 11	1.69 $\pm$ 0.20	315	812	3.2
	HF2	-----	256 $\pm$ 59	1.45 $\pm$ 0.10	128	870	5.1
	HF4	----Q---	238 $\pm$ 19	1.83 $\pm$ 0.05	119	790	4.8
	Pst	----HE--	75 $\pm$ 1	0.053 $\pm$ 0.005	375	-14	1.5
	Xho	----LE--	127 $\pm$ 3	0.070 $\pm$ 0.006	635	-7	2.5
	Q6E	-----E--	25 $\pm$ 2	0.054 $\pm$ 0.003	113	-12	0.5
		12345678					
AB4	WT	QVQLQSG	462 $\pm$ 14	2.22 $\pm$ 0.06	116	1438	2.3
	PH1	-----	153 $\pm$ 30	2.45 $\pm$ 0.16	38	683	0.8
	PH2	NI--E---	140 $\pm$ 35	1.71 $\pm$ 0.25	35	615	0.7
	HF2	-----E---	212 $\pm$ 17	2.61 $\pm$ 0.01	53	1222	1.1
	HF4	-----	399 $\pm$ 59	1.64 $\pm$ 0.31	100	456	2.0
	Pst	-----E--	3.2 $\pm$ 0.3	0.053 $\pm$ 0.002	16	-13	0.06
	Q6E	-----E--	9.3 $\pm$ 1.6	0.067 $\pm$ 0.006	47	11	0.9
		12345678					
AB3	WT	EVQLQSG					
	PH1	Q---E---	30 ^e	2.75 $\pm$ 0.01	75	382	0.2
	Q6E	Q---E---	0.2 ^e	0.065 $\pm$ 0.006	1	-8	0.01

^aThe clones AB2-PH2 and -HF2 and AB4-PH1 and -HF4 and AB4-Pst and -Q6E differ from each other by the other primer encoded FRs.

^bThe amount of scFv was determined on Western blot using a standard curve of purified scFv.

^cFor ELISA plates were coated with 4 $\mu\text{g/ml}$ hCG; background signals were in the 0.05 - 0.08 range.

^dBIAcore experiments were performed with a high density hCG surface (4369 RU) (see Figure 3). The hCG-reactivity was determined at time point t=400 s.

^eSingle observation.

II), which after sequencing were found to differ from the first batch of scFvs only in the oligonucleotide-encoded regions of V_H - and V_L -FRs. Constructs containing the complete wild type sequence (AB2/4-WT in Table II), based on sequence analysis of the V-genes including the Ig-leader (see Materials and methods), and cloned after amplification with wild type FR-specific primers, also expressed functional scFv fragments. Thus the non binding of the first scFvs, AB2/4-Pst and AB2/4-Xho, must have been caused by one or more mutations in one of the FRs of V_H or V_L , introduced by the oligonucleotides used for the amplification.

The fact that this happened in two murine antibodies which recognise different epitopes on the same antigen was sufficiently intriguing to prompt us to search for the mutation responsible for loss of antigen binding. We only changed the V_H -FR1 region of clones AB2/4-Pst and AB2/4-Xho into wild type. Functionality was now regained as indicated by ELISA (AB2-HF2 and AB4-HF4 in Table II). Alignment of the V_H -FR1 encoded primer sequences of the functional and non functional clones as shown in Table II revealed two candidate residues for causing the problem: the residues at positions 5 and 6. Mutants of AB2 made with the V_H -FR1 sequence of AB4 (coded AB2-HF4) and of AB4 with the V_H FR1 sequence of AB2 (coded AB4-HF2) were functional, leading us to exclude residue 5. The involvement of the only remaining residue, E at position 6, was confirmed by the generation of antigen binding deficient scFvs with wild type residue glutamine of position 6 substituted by glutamate (clones coded AB2/4-Q6E in Table II). These mutants were indeed inactive in ELISA.

A third hCG antibody, coded AB3, was also non-functional when the wild type residue number 6 glutamine was mutated into glutamate (Table II).

4.2 Analysis of hCG reactivity in ELISA and BIAcore

We first analysed the effect of the antigen-binding loss mutations on the total production of the scFvs. The Q6E mutant scFvs were tested using periplasmic preparations and compared with the other scFvs, for binding in ELISA (Table II), and for production by Western blots (Figure 2).

Fig. 2. Western blot detection of the scFvs produced by the different AB2 clones with the anti-FLAG-antibody M2.

Panel A : periplasmic fractions, lane A 0.5 μ l (0.14% of a 35 ml culture) and lane B 2.5 μ l (0.71%) periplasmic fraction; *Panel B :* total cell protein (the cells from 0.5 ml culture were boiled in 200 μ l Laemmli sample buffer); lane A 1 μ l (0.007% of a 35 ml culture) and lane B 5 μ l (0.035%) of the sample. The obtained signals were compared with three different amounts of affinity purified scFv (5, 25, 50 ng; right hand lanes).

The concentration of scFv was determined by comparison of the measured sample signal with the signal of a range of standards (AB4-PH2 purified on an antigen column and quantified with OD₂₈₀) present on the same blot.

First of all, there were no real differences between the total production levels, i.e. the sum of insoluble and soluble scFv (Figure 2, panel B), of non-functional (AB2-Pst, AB2-Xho, AB2-Q6E) and functional clones (AB2-WT, AB2-HF2/4, AB2-PH1/2), thereby excluding variations in mRNA levels or translation. However, examination of the soluble scFv, as was found in the periplasmic fractions, revealed that the E6-containing mutants in general produce lower amounts (Figure 2, panel A; Table II).

A possibility is that the mutation influences the affinity of the antibody, and that binding would be reduced to a level not detectable by ELISA; therefore the amount of soluble scFv used in the ELISA tests was scored. All non binding scFvs gave no-binding, background level signals at scFv concentrations as high as 115-635 ng/ml (AB2) and 16-260 ng/ml (AB4). The enormous difference in ELISA suggests that these scFvs have lost all binding completely; for example active clone AB2-HF2 gave a signal of 29 times background level binding at a concentration of 128 ng/ml, contrasting inactive clone AB2-Xho, where no binding was detected even at scFv concentrations of 635 ng/ml.

To test whether the kinetics of binding had been modified by the Q6E mutation, for example a dramatic shift in the off-rate had been induced, the scFvs were tested in BIAcore using hCG immobilized on the Sensorchip (Figure 3). The ELISA results (Table II) were confirmed in terms of reactivity against antigen: all V_H-FR1 variants which scored negative in ELISA, did not react with hCG in BIAcore either at all. The measurement should detect any residual reactivity of the scFvs produced by the Q6E variants. Again the difference is striking: of the reactive clone AB2-WT the surface captures 320 RU, when scFv was injected at a concentration of 1.8 µg/ml, while AB2-Xho scores only -7 RU, even at scFv concentrations of 2.5 µg/ml. To exclude that the immobilisation of antigen on the sensor chip surface itself had differentially altered the epitope on hCG and therefore its affinity and kinetics of binding by the scFv variants, the binding analysis was carried out also with indirectly immobilised scFv fragments. Anti-FLAG antibody M2 was coated and used to capture scFv fragments in periplasmic extracts as is shown Figure 3B. Upon injection of hCG, a significant rise in signal was obtained for clone AB2-WT, but for the inactive clone AB2-Xho the signal returns to its level reached before injection; this while the amount of captured scFv of clone AB2-WT is lower than for AB2-Xho. In this assay, non-binding was confirmed for all ELISA-negative clones (results not shown). Thus the Q6E mutation causes complete loss of antigen binding in two unrelated antibodies binding to different epitopes of hCG, hinting towards a common cause beyond effect on antigen binding.

In order to exclude problems caused by the single chain format itself, we constructed Fab-producing clones (Hoogenboom *et al.*, 1991; Carter *et al.*, 1992) from AB4, with or without the Q6E mutation. The wild type Fab reacted in BIAcore and ELISA while the Q6E mutant failed to

do so (results not shown), proving the effect of the mutation on antibody expression is format independent. Thus the reactivity problems of these antibodies are related to the primary sequence of their V_H domains.

Fig. 3. Binding analysis of the reactivity of the scFvs against hCG using surface plasmon resonance (BIAcore). (A) Overlay plot of binding profiles of three AB4 derivatives on a high density coated hCG sensor chip (4369 RU hCG immobilised). At $t=100$ s the periplasmic fraction was injected; the linear relation between response and time indicates that the association is diffusion limited (Jonsson *et al.*, 1991). At $t=340$ s the surface was washed with Hepes-buffered saline (HBS) buffer. (B) Overlay plot of three AB2 derivatives. ScFv was injected at $t=100$ s on a high density coated anti FLAG M2 chip (9667 RU). After washing, which started at $t=360$ s, antigen binding was analysed by injecting hCG (concentration of 15 $\mu\text{g/ml}$) at $t=570$ s. The amount of captured hCG can be deduced during the following washing cycle starting at $t=850$ s. The amount of captured hCG is the difference between the timepoint before hCG-injection ($t=550$ s) and at the start of the second buffer wash ($t=860$ s). (C) Overlay plot of three AB2 derivatives injected at $t=100$ s on a high density coated hCG-chip (4369 RU hCG immobilised). At $t=80$ s periplasmic fraction (1:1 diluted with HBS) was injected; on $t=360$ s the dissociation phase started by washing the surface with HBS-buffer.

Often antibody fragments produced in bacteria mis-fold, and active materials may be retrieved by *in vitro* refolding. Refolding as described previously (Glockshuber *et al.*, 1992) of the aggregated scFv resident in the spheroplasts of the Q6E scFvs improved the yield of soluble scFv protein, but did not lead to any antigen binding product. The efficiency of the refolding procedure was shown by applying it on aggregates of functional clones. High yields of refolded antigen binding scFvs were obtained as was measured by ELISA, BIAcore and by quantitative isolation of scFv on antigen columns (results not shown). Thus the Q6E mutation must affect both the *in vivo* and *in vitro* folding dramatically, in such way that no antigen-binding material can form.

In conclusion we can state, that all attempts to detect antigen binding of the E6-mutant scFvs or Fabs in ELISA or BIAcore failed, in spite of the fact that the amount of soluble fragment should be sufficient for yielding positive signals. Even increasing the amount of soluble scFv by refolding did not improve the test results. These findings indicate that the mutation causes a complete loss of binding affinity.

4.3 Analysis of conformation by trypsin digestion

The integrity of Q6E and wild type of AB2 and AB4 was examined by trypsin digestion of the soluble material, as done before with AB-fragments (Glockshuber *et al.*, 1990) or with β -lactamase (Minsky *et al.*, 1986). The wild type and the Q6E mutant scFv of the two different ABs were mixed in equal amounts. After incubation with trypsin both types of AB fragments were separated by electrophoresis and detected on Western blot with anti FLAG AB (Figure 4).

Fig. 4. Trypsin accessibility experiment on wild-type and Q6E mutant scFv fragments derived from AB2 and AB4. Periplasmic extracts were mixed in such a way that an equal amount of each scFv was included in the digestion reaction. Trypsin addition is indicated with + and -; the samples of lanes 2 and 5 were incubated with 0.2 mg/ml trypsin, those from lanes 3 and 6 with 40 mg/ml. Lanes 4 and 7 are controls (no digestion). The reactions were stopped by adding SDS-PAGE loading buffer and analysed on 15% polyacrylamide gels to separate the AB2 and AB4 scFvs. After blotting, scFv was detected with anti-FLAG. As a positive control AB4-WT is shown in lane 1.

The two Q6E mutants are more rapidly degraded by trypsin than the wild-type proteins. Thus the Q6E mutants are much more protease sensitive, indicating misfolding, while the functional scFvs

are relatively well protected against protease due to their correctly formed immunoglobulin fold. In addition the amount of soluble scFv produced by the non functional clones is reduced when compared to the functional versions (and sometimes quite dramatically, e.g. the Q6E mutants of AB2 and AB4 in Table II), however, soluble fragments are still produced. Since unfolded material would be normally present as aggregate, the protease sensitive Q6E protein found in the soluble periplasmic fraction must be misfolded, explaining the complete absence of binding affinity, but it is not aggregated. Figure 5A shows a hypothetical model for folding of the functional scFvs, in which intermediary A can not be detected since it is rapidly transformed into the native (active) state or in the aggregated state. In the Q6E mutant the folding pathway to this end product is blocked, and, besides a lot of aggregated protein, a protease sensitive, non-binding protein is formed (Figure 5B).

Fig. 5. Hypothetical model of the different folding pathways that are followed by the VH domains of the various antibodies studied.

(A) In antibodies with IIA-VH sequences with the wild-type Q6 the formation of the correct folding intermediary A is promoted. Although part of the protein is lost due to aggregation, the majority can be found as soluble and antigen binding molecules. (B) Mutation of Q to E at position 6 prevents formation of folding intermediary A and leads to an alternative intermediary, B, with enhanced propensity for protease cleavage and aggregation. The intermediate may be found as a soluble protein, but cannot bind antigen.

(C) Additional mutations in FR1 restore the stability of folding intermediary A, and thus production of correctly folded antigen binding antibodies. Residue 6 is indicated with a small black (Q) or grey (E) circle; compensating mutations in FR1 are indicated with an open white circle (in C). Putative β -strands are indicated.

4.4 Compensating mutations for the E(6) mutant in V_H of AB2 and AB4

Analysis of the structures did not reveal any eye catching differences between related antibodies with either Q or E at position 6 in the immediate vicinity. In the murine antibody directed against 2,2,6,6-tetramethyl-1-piperidinyloxy-dinitrophenyl (coded IBAF; Leahy *et al.*, 1988), of which the structure has been solved by X-ray diffraction, residue 6 (E) is positioned between and may interact with residues from β -strands B, F and G (Figure 6A and 6B). First, aspartic acid at position 96 (Kabat numbering (Kabat *et al.*, 1991)) was converted into asparagine in AB2; secondly lysine at position 23 was substituted by alanine. Neither mutation affected antigen binding in the wild type construct (Q6); however, when introduced in the mutant (E6) version, they did not result in the production of functional scFv.

Since this approach guided by the putative interaction of residue 6 with other variable domain residues did not offer any other hints, we changed our approach by looking at sequences from antibodies which naturally harbour glutamate at position 6. The studied anti-hCG antibodies possess variable heavy chain regions belonging to subgroup IIA (Kabat *et al.*, 1991). 97% of the known IIA antibodies have glutamine on position 6, indicating that it is highly conserved. In contrast, subgroup IA antibodies use mainly glutamate at position 6 (99%). This family-dependent conservation is remarkably well visible in human V_H genes also (Tomlinson *et al.*, 1992). We aligned the sequences of the V-regions of the hCG antibodies, together with a IIA antibody having a glutamate residue, which is coded MBR1 (Orlandi *et al.*, 1989), and TFS-129, a V_H-Class IA antibody (Figure 1). The main differences within the aminoterminal part are on position 13 (lysine present in the anti hCG antibodies and in IIA, versus serine in MBR1), 19 (lysine in IIA versus threonine in MBR1 or serine in IA) and 20 (methionine or isoleucine in anti hCG's versus leucine in MBR1 or IA). We changed all three residues within AB2 into their counterparts of MBR1 (K13S; K19T; M20L) again in the wild type construct AB2-WT ('Q6'), and in the mutant AB2-Q6E ('E6'), yielding 'MUT1' versions (Table III). The amount of soluble scFv present in the periplasmic fractions was determined on Western blot, while the hCG reactivity was tested in BIAcore and ELISA (Table II). As shown in BIAcore (Figure 3C) and ELISA, antigen reactivity was obtained with the periplasmic fraction of the E6 triple mutant (containing the three mutations located within the FRI-region). In hCG ELISA a signal of 6 times background was obtained at a concentration of 1650 ng/ml and of 1.5 times background at dilutions of 165 ng/ml. For comparison: the Q6 triple mutant gave signals of 23 times background measured at a concentration of 187 ng/ml, indicating that this antibody fragment has a higher affinity or, most likely, that a smaller fraction of the 'E6 triple mutant' consists of active protein. A higher signal was found also in BIAcore for the Q6 triple mutant compared to the E6 triple mutant (respectively 679 versus 195 RUs; see Table III).

(A) **(B)** **(C)**

Fig. 6. (A) Presentation of the structure of antibody 1BAF made with the program Sybyl (Tripos) showing the location of residue number 6 (Glu) in relation to the intradomain disulfide bond. (B) A detailed picture of the position 6 residue of V_H and its neighbouring residues Thr23, Trp96, Gln105 and Gly106. (C) As B in combination with the residues targeted for mutagenesis (Lys13, Ser19 and Leu20). α -trace representation of the light (green) and heavy (yellow) chains of the Fv-domain. These images illustrate the buried position of residue 6 between the N terminus of the H chain and the β strands preceding and following CDR H3 (purple). The side chain atoms of Glu 6 (carbons in orange, oxygens in red) and of the backbone NH-groups surrounding the buried sidechain (e.g. those from Cys92, Gln105, Gly106 and Glu6 itself with nitrogen in blue and hydrogen in white) are depicted as full Vanderwaals spheres.

However, when only a single mutation was introduced, threonine on position 19, the effect was less profound than the combination of the three mutations (Table III; K19T-mutants). It thus appears possible to restore folding of the Q6E versions of these IIA antibodies by engraftment of FR1 residues that are normally found in conjunction with residue E at position 6.

Table III. V_H-FR1 mutations compensating for loss of binding in Q6E variants.

AB	Clone	Mutations	Prod µg/L	ELISA ^a		BIA ^b RU	ahCG µg/mL
				OD ₄₅₀	µg/L		
AB2	Q6-MUT1	K13S, K19T, M20L	75±6	1.68±0.05	187	679	1.9
	Q6E-MUT1	K13S, K19T, M20L	72±8	0.41±0.07	1815	195	1.7
	Q6-MUT2	K19T	87±16	2.85±0.02	218	639	2.2
	Q6E-MUT2	K19T	23±1	0.13±0.01	224	44	0.6
	Q6 (WT)		97±13	0.76±0.18	74	840	2.5
	Q6E		21±2	0.065±0.004	536	5	0.5
AB4	Q6-MUT2	K19T	100±18	1.31±0.23	13	1032	2.5
	Q6E-MUT2	K19T	32±4	0.33±0.02	322	84	0.8
	Q6 (WT)		123±32	0.89±0.13	61	1051	3.1
	Q6E		9±3	0.068±0.001	235	11	0.2

^aELISA was performed with plates coated with 10 µg/ml hCG. ^bFor BIAcore the hCG-reactivities were determined on a high density coated chip (4369 RU) at t=400 s.

5. Discussion

We demonstrate the importance of the wild type residue glutamine on position 6 of V_H domains in three anti-hCG monoclonal antibodies recognizing different epitopes on the antigen. Introduction of glutamate, encoded by cloning site *Xho*I in the FR1 region, abolishes binding activity, in spite of the fact that the introduced residue is also naturally occurring at this position. In addition to the antibodies described in detail here, 3 more antibodies (one additional anti-hCG and two anti-HIV-1 p24 antibodies) with a V_H of class IIA were analysed; in all cases the cloned scFv versions with Q6E were not reactive. Therefore we believe that this phenomenon is a general feature for murine antibodies with a V_H-domain of the IIA class.

How does the glutamine residue of position 6 exert its effect on the reactivity of the anti-hCG antibodies? In all clones tested, we have found a complete loss of antigen binding. Since antibodies recognising completely different antigens or epitopes showed the same defects upon

mutation, we suspect that residue 6 is not directly or indirectly involved in shaping and/or influencing the antigen binding site.

One possibility for the dramatic effect of the residue 6 point mutation would be the effect of the introduction of a charged residue within close proximity of the leader peptide, which might disturb transport over the membrane into the periplasmic space (Ayala *et al.*, 1995). The residue 6 point mutation, however, involves an evolutionary conserved change: in murine and human antibody germline segments, either Q or E is used. From inspection of the sequences of germline V-genes available in databases, the choice appears to have been made in a nearly family-dependent way, with very few exceptions. In addition, in somatically hypermutated antibodies other residues at this position are very rare. Finally, we and others have selected functional scFvs or Fabs which are actively secreted by the bacteria and form antigen binding antibody fragments, with a glutamate on position 6, making this hypothesis highly unlikely.

Our data suggest that incorporation of glutamate at residue 6 in the V_H-FR1 of IIA antibodies prevents folding of the V_H into its native immunoglobulin conformation, but leads to an alternative, inactive and protease susceptible version (Figure 5). This idea is supported by the presence of the lower amounts of soluble scFv expressed by the inactive mutants, as has been found in some other antibody variants with dramatic effects on folding (Glockshuber *et al.*, 1992). The antibody folding is disturbed in the mutant protein, thereby making it more susceptible for activity of trypsin *in vitro* and bacterial proteases *in vivo*, as was seen before with antibody fragments (Glockshuber *et al.*, 1992) and a T-cell receptor (Wulfig and Plückthun, 1994). When producing the scFv by *in vitro* refolding, soluble material is retrieved, however, antigen binding is never restored, indicating that the *in vivo* and *in vitro* folding process has been interfered with, and other causes of non-functionality such as differential interactions with chaperonins, effects on secretion, or aggregation rates can be excluded. Our data indicate that glutamine in antibodies with a V_H of the class IIA is crucially involved in the folding of the antibody V-domain, and that its exchange to glutamate interferes with folding of most if not all IIA antibodies, independent of the nature of the antigen bound by the antibody. Since this is the case for *in vitro* refolded protein as well, we suggest that the folding pathway (Figure 5A) has been re-routed by the Q6E mutation, thereby completely disturbing the production of correctly folded protein. The alternative pathway leads to mixes of aggregates, which are rapidly destroyed by bacterial proteases *in vivo* (Figure 5B), and non-aggregated, misfolded protein. This effect may be caused by destabilisation of the transition state nucleus, for example by an increase in the time in which hydrophobic areas are transiently exposed, and leads to entrapment of intermediate folding states. The effect of the mutation is, however, unique, in that it induces a complete block in the production of correctly folded protein.

The structural role of the position 6 residue in immunoglobulin domains was described by Lesk and Chothia (Lesk and Chothia, 1982), although the authors confined their discussion to the residue present in murine light chain variable regions and constant domains, which is

invariably glutamine. The side-chain of residue 6 is oriented towards the hydrophobic core of the protein, and for V-regions at least, the side chain atoms of the residue form hydrogen bonds to the main chain atoms of the opposite β -sheet, ie. with the crucial cysteine residue on position 88, with tyrosine 86 and threonine 102. Due to the interruption of the normal β -sheet association by the bulky amide group of glutamine 6, an extra residue was inserted in the opposite strand thereby creating a β -bulge fitting around this amide group. We observed no variation in the localisation of the side chain of residue number 6 or in the formed hydrogen bonds between heavy chain V-regions with either glutamine or glutamate of murine origin, and therefore speculated about possible differences in stabilizing interactions with other residues. Alignment with a rare member of subgroup IIA having glutamate on 6 (MBR1) revealed a number of differences within the FR1 region (spanning the first β strands A/A' and B), which upon grafting onto an inactive Q6E-IIA antibody, restored antigen binding activity partially. We suggest that the formation of a salt bridge between E6 and K19 in the inactive antibody, interferes with the local folding of the anti-parallel β strands A/A' and B of FR1; this is restored in the E6, T19 version. However, more residues must be involved in this folding intermediate, since regained activity is only partial. This hints towards the involvement of the anti parallel β strands of FR1 in an important folding intermediate or subdomain, which may be composed differently in class IIA and IA antibodies (Figure 5C). The involvement of more residues suggests that the number 6 residue might be a constituent of a folding nucleus (Shakhnovich *et al.*, 1996). In this folding model just a limited number of residues of a protein form most contacts in the transition state. Regarding the significance of the residues for the total folding process, it was suggested that the residues of the folding nucleus should be evolutionary conserved. Mutations affecting the stability of the transition state nucleus should have a greater effect than other mutations. In this respect the V_H number 6 residue of the murine antibodies meets the criteria of conservation and the effect of the mutation. It was already noticed (Kirkham and Schroeder Jr., 1994) that the two solvent exposed regions within FR1 (codons 6-24) and in FR3 (codons 67-85) are highly conserved between members of a single V_H-family, but divergent between members of different V_H-families. Our data suggest that these areas might form part of a family specific folding nucleus, and that they, when completely defined, may be grafted between antibodies from different classes. However, it remains to be experimentally confirmed that grafting completely restores the "active" conformation by for instance the determination of trypsin sensitivity or by CD spectroscopy.

Recently Plückthun and colleagues described the presence of a folding nucleus in the inner β -sheet of the V_L of a scFv derived from McPC603 (Freund *et al.*, 1996), but found no proof for a similar nucleus in the V_H. In the antibodies that we have isolated such crucial folding nucleus may exist in the V_L also, and the V_L may also 'chaperone' the folding of the V_H as suggested by Plückthun. However, since V_H-FR1 residues do not display any side chains that become buried upon V_L - V_H association (Freund *et al.*, 1996), it is unlikely that the role of FR1 in V_H folding is

to merely provide a surface for V_L binding to assist further V_H folding. It is more likely that by the interaction between the V_H FR1-residues of β strands A/A' and B an early V_H folding nucleus may be formed independent from the V_L folding nucleus, and that the V_L may assist in further folding of the V_H -domain.

For class IIB antibodies, the effect of mutation of V_H residue 6 on antigen binding seems to be different. This was observed by Kipriyanov and colleagues (Kipriyanov *et al.*, 1997). They also found a severely lowered production level of soluble Q6E mutant scFv, what was already indicated in our earlier work (De Haard and Kazemier, 1995), in fact comparable to what we observed, but its affinity was identical to the wild type fragment. If the affinities of our Q6E mutant antibody fragments derived from the IIA antibodies AB2 or AB4 would be identical to those of the wild type fragments, we would have expected high responses in ELISA and BIAcore when used at equal concentrations as the wild type derivatives. Therefore, our data suggest that for folding, IIA antibodies require absolute conservation of residue 6, while the studied IIB antibody is more tolerant to mutation of this residue.

Besides obvious implications on antibody expression in bacteria and possibly other expression systems, our findings will have an impact on the construction of phage antibody libraries. Mostly phage antibody repertoires are derived from immune or naive B-cells, using oligonucleotides based in the FR1 and FR4 regions of the light and heavy chain V-genes (for review see Hoogenboom *et al.*, 1992b). For amplification of murine V_H genes, the frequently used degenerate primer VH1BACK (Ward *et al.*, 1989), which primes in FR1 covering the first 24 nucleotides, allows incorporation of either Q or E at position 6. Although preferentially the naturally occurring residue will be amplified and not the mismatched (non-WT) version, it cannot be excluded that murine IIA V_H genes are supplemented with E rather than Q at position 6, yielding non-functional antibodies. Problems caused by primers, which incorporate non wild type residues, will also arise when using 'family-specific' primers for the amplification of rearranged V-gene pools from naive or immune B-cells (Marks *et al.*, 1991). Extensive sets of family specific primers for the amplification of murine V-genes have now been described by several groups (Orum *et al.*, 1993; Krebber *et al.*, 1997), but combining them within a single PCR reaction might promote mismatching and thereby decrease the number of functional displayed antibody fragment within a library. Future primer design and -choice should take into account the incorporation of 'original' residues in V_H -FR1 (as may be done for synthetic antibody libraries; Hoogenboom and Winter, 1992a), to retain essential elements of the folding nucleus, which will contribute to the creation of antibody libraries of optimal quality.

6. References

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CHAPTER 2

A FR1 MUTATION IN VL AFFECTING ANTIGEN BINDING

In press

Residue 4 in the Vernier Zone of mouse antibodies with a subgroup II
kappa light chain is a critical determinant for antigen recognition

*Hans J.W. de Haard, Bert Kazemier, Arie van der Bent, Peter Oudshoorn,
Piet Boender, Jan-Willem Arends, and Bob van Gemen*

Immunotechnology

1. Abstract

Background: During the conversion of murine monoclonal antibodies directed against the human chorionic gonadotropin (hCG) into bacterially expressed single chain fragments (scFv), we found a major reduction of binding activity upon introduction of a primer encoded mutation. **Objectives:** In this study we tried to determine which mutation was responsible, and on what manner this mutation affected antigen binding (structural effect versus direct involvement of the residue in binding). **Results:** No binding could be detected, when the wild type residue methionine at position 4 within the Framework region I of the VK light chain was substituted by serine in two antibodies with a subgroup II kappa light chain. However, a similar replacement within an anti-hCG antibody with a subgroup IV kappa light chain, and thereby having leucine as wild type residue, did not affect the binding characteristics. The mutant scFv's derived from both AB-s sensitive for substitution by serine never reacted with antigen in ELISA. By using Surface Plasmon Resonance, a residual binding was observed only under conditions, when a high density antigen coating was applied; however, an increased dissociation, relative to that of the wild type scFv, and the absence of reactivity in ELISA suggest a drastically altered affinity. **Conclusion:** A structural explanation for the changed binding characteristics can be the influence of the position 4 residue, as being a constituent of the Vernier Zone, on the position of the CDR1 loop of VK, which might harbour residues that directly bind to antigen, or indirectly positions other variable loops of the binding pocket. An increased sensitivity for trypsin digestion supported the hypothesis of a local conformational change in the serine mutant of the subgroup II kappa containing antibody.

2. Introduction

The binding part of an antibody consists of the amino terminal domain of the heavy (VH) and light chain (VL), which are closely associated by non-covalent interactions. The relatively conserved framework residues (FR) of the variable domains are involved in the formation of a nine-stranded antiparallel β barrel conformation. The three hypervariable loops of each domain, forming the complementarity determining regions (CDRs), connect the β -strands at the aminoterminal end of the barrel and harbour the antigen contacting and binding residues.

Besides providing a scaffold for mounting the CDR-loops, FR residues occasionally make direct contacts with antigen (Sheriff *et al.*, 1987; Padlan *et al.*, 1989; Bentley *et al.*, 1990), or on the other hand, they can affect binding indirectly by positioning of the CDR (Chothia *et al.*, 1989; Tramontano *et al.*, 1990). Chothia and Lesk (1987) described the influence of particular framework residues on the main chain conformation of the hypervariable loops. The defined set of canonical structures of CDR loops seems to be determined by the size and the presence of

well defined residues at particular sites within the loop but also in the framework regions (Chothia *et al.*, 1989). During the humanisation of antibodies, where murine CDR loops are grafted on a human framework, it is often necessary to include a number of murine framework residue(s) for retaining the parental affinity (Riechmann *et al.*, 1988). By making key contacts with CDRs these framework residues help to maintain the conformation of the loops. The critical residues, the so called Vernier residues, are often located on the borders of the CDRs and were analysed in more detail (Foote and Winter, 1992).

For the production of antibodies or fragments thereof in mammalian cells or in bacteria, the variable region (V-) genes are amplified by the polymerase chain reaction (PCR) with "universal" primers suitable for the isolation of a range of heavy and light chain sequences. The design of primers in the FR4 region is relatively easy, since this area is encoded by a limited number homologous joining (J-) segments. However, FR1 primers are family specific and have to be used separately (Marks *et al.*, 1991) or were synthesized as degenerate oligonucleotides (Orlandi *et al.*, 1989; Sastry *et al.*, 1989). Restriction sites, rarely occurring in natural antibody variable sequences, were often introduced to facilitate cloning (Sastry *et al.*, 1989; Orlandi *et al.*, 1989; McCafferty *et al.*, 1990). As a consequence of degeneracy and of the introduced sites, the primer encoded regions will contain non wild type residues, and especially for the amino terminal regions this might be critical for the binding characteristics (Padlan, 1991).

We already observed the drastic effect of an evolutionary conserved substitution of the position 6 residue in VH during the conversion of two different mouse antibodies recognizing the human chorionic gonadotropin (hCG) into bacterially expressed scFv antibodies (de Haard *et al.*, 1998). When the wild type residue glutamine, present in antibodies with a subgroup IIA heavy chain (Kabat *et al.*, 1991), was mutated into glutamic acid, mainly found in class IA heavy chains, antigen binding was completely lost as the result of misfolding.

Here we describe the effect of a mutation within the amino terminal region of the kappa light chain caused by the introduced restriction site *Sa*II. When the position 4 residue methionine of two anti-hCG antibodies with a subgroup II kappa light chain was replaced by serine, antigen binding was abolished completely as was measured in ELISA. In contrast, one other hCG antibody with a class IV kappa chain tolerated the introduced serine. Analysis of the structure of the mutant antibodies (AB-s) by trypsin digestion suggested that a local distortion of the conformation might be responsible for a drop in affinity.

3. Materials and methods

3.1 Cloning and screening

The murine monoclonal antibodies (IgG1, kappa) used for the conversion into bacterially produced scFv's are coded AB2, AB3 and AB4 and recognize the β -core fragment, the β -

Carboxy Terminal Peptide (CTP), and β -core fragment of the gonadotropin hCG (nomenclature see Birken *et al.*, 1990). The amplified variable regions were cloned in the phagemid vector pVI, which is identical to pHEN1 except with a FLAG- (Hopp *et al.*, 1988) instead of the Myc-tag. More experimental details including the sequences of the used primers can be found elsewhere (de Haard *et al.*, 1998).

Briefly, the antibody V-regions were initially cloned with degenerated primers containing **restriction sites** introduced within the variable regions (a *XhoI*- or *PstI*-site in the VH FR1, *NcoI* in VH FR4, *SalI* in the VK FR1 and a *BclI*-site within VK FR4). After electroporation (Dower *et al.*, 1988) in the *E. coli* non suppressor strain TOP F', individual clones were screened with PCR using the M13reverse and the geneIII-primer. Transformants containing the expected inserts were grown in 50 ml medium and induced (Marks *et al.*, 1991). The scFv containing periplasmic fractions, prepared by the shock procedure with borate buffered saline (Skerra and Plückthun, 1991), were analysed in ELISA and surface plasmon resonance for binding to hCG (for experimental details see (de Haard *et al.*, 1998). Kinetic analysis was performed with surface plasmon resonance using the BIAcoreTM; CM-chips (Biacore AB, Lund, Sweden) were applied for covalent coupling of hCG. Experiments were performed with a high density coat of hCG (4369 response units (RU); molecular weight of 45 kD).

The **wild type constructs**, encoded WT, were made by amplification with a VH FR1 primer extended into the pelB leader sequence and containing a *SfiI*-site, while the VK FR4 primer with a *NotI*-site was extended to the FLAG-sequence as described (Clackson *et al.*, 1991). The heavy and light chain V-genes were assembled with the linker fragment in a splicing by overlap extension polymerase chain reaction (SOE-PCR) (Horton *et al.*, 1989) and cloned as *SfiI/NotI*-fragments. TOP F' transformants were screened by PCR as above and used for the expression of soluble scFv.

A similar cloning strategy was applied for the generation of **phage libraries**. Again, amplification was performed with extended primers containing the *SfiI*- and *NotI*-sites, which were degenerated to some extent. After transformation of the suppressor strain JM101, phagemid particles were rescued as described (Marks *et al.*, 1991). After two panning rounds on purified hCG, coated at 3 μ g/ml on polystyrene plates, clones (encoded PH) producing functional scFv were detected by ELISA using the culture supernatants. Phage was prepared from functional clones and was used for transfection of TOP F' cells. The resulting transformants were grown and induced as described before.

Mutants AB4-V2I and AB4-M4S were constructed by application of SOE PCR with primers encoding the mutation and having an introduced diagnostic (silent) restriction site; the number of cycles was limited to 20. The scFv encoding inserts of the clones characterised on binding were completely sequenced.

3.2 Trypsin digestion

The stability of the scFv produced by the serine mutant of AB4 (AB4-M4S) could be directly compared with the wild type scFv of AB2 by combining both periplasmic fractions, containing approximately 50 ng antibody fragment, into a single digestion reaction containing 100 mM Tris-HCl pH8.0 and 10 mM MgCl₂ and 200 or 40 µg/ml (ICN Biomedicals Inc, Costa Mesa, CA, USA). Alternatively, a mixture was made of the extracts from clones AB4-M4S and AB2-Q6E. After 2 hours incubation at 16°C the digestion was stopped by adding Laemmli buffer. The scFv fragments derived from the two different AB-s were separated on a 12% polyacrylamide gel. After blotting the products were detected with anti-FLAG M2 antibody (Kodak IBI, New Haven, USA).

4. Results

4.1 Identification of a VK-FR1 mutation affecting antigen binding

A panel of murine anti-hCG mABs, of which the light chain sequences are shown in Fig. 1, was converted into bacterially expressed scFv's. Initially the V-regions were amplified and cloned with primers containing introduced restriction sites. For the antibody coded AB2, which is reactive against the β core fragment of hCG, problems were encountered with respect to the production of functional scFv. This clone was analysed in more detail. Individual primer encoded FR-regions were exchanged to wild type, and it appeared that when the mutant Vk FR1 was replaced, the produced antibody fragments were reactive in ELISA against hCG (Table I).

		Light chain									
Antibody Subgroup		FR1		CDR1	FR2		CDR2	FR3		CDR3	FR4
		10	20	TARDCE 30	40	50	60	70	80	90	SA 100
#4	II	GVVMTQTPLSLFVSLGDOASISC	RSSEGLYLNKNTYLH	WYLGKFOGSPKLLIY	EYENRLE	GVFDFSGSGSGTHFTLKISVVEAEDLGVYFC	SGSTHVP WT	FGGQTELEIKR			
#3	II	-----N-S-TI-QP-----	K---T-LYG--K---N	L-R-----R---	L-KLD-	-----T-----D-----N-----L-Y-	M-G--F- H-				
4-4-20	II	-----VSSQ-----R		V-----V---	P-----	-----D-----					
#2	IV	KIVLTQSPAIMSASLGRVYNTC	TASSS	VSSSYLN	NYQQKTGSEPKLMTY	STENLAS	GVFANFSGSGSGTSEVLTISMEAEADAATYTC	HCYHRSP YA	FGGQTELEIKR		
AN02	IV	Q-----P--K-----	S---	VYTHY	-----P---R-L-	D-----	--V-----R-----	Q-NSYFPIT	--V-----L--		

Figure 1. Deduced amino acid sequences of the variable light chain regions of anti-hCG monoclonal antibodies AB2, AB3 and AB4. Numbering of the residues and light chain classification was performed according to Kabat *et al.* (1991). In addition the Vk sequences have been aligned of the anti-2,2,6,6-tetramethyl-1-piperidinyloxy-dinitrophenyl antibody AN02 (panel above) and the anti-2-methyl-2,4-pentanediole antibody 4-4-20 (panel below).

This region was examined more closely. Only two mutations could be responsible: at position 2, where the wild type valine was replaced by isoleucine, and at position 4, where methionine was substituted by serine. The activity of two new variants, with the complete wild type sequence except on position 2 (clone V2I) or 4 (clone M4S), showed that serine on position 4 is crucial for antigen binding (Table I).

Another antibody coded AB3, directed against the carboxy terminal part of the β subunit of hCG, and which shares the V κ -family (class II) with AB4, was analysed in a similar way. Here also the mutation M4S abolished antigen reactivity as measured in ELISA (results discussed below). It is interesting to see that AB2, reactive against hCG β core fragment and with a V κ -region of a different family (class IV), tolerates the substitution of its wild type residue leucine by serine (see results below).

Table I. Determination of the additional primer encoded FR-region in AB4 responsible for loss of antigen binding.

Clone	VH ^a		V κ ^a		ELISA ^b
	FR1	FR4	FR1	FR4	
HF4-A	WT	MUT	MUT	MUT	-
HF4-C	WT	MUT	MUT	WT	-
HF4-D	WT	WT	MUT	WT	-
HF4-B	WT	MUT	WT	MUT	+
HF4-E	WT	MUT	WT	WT	+
V2I	WT	MUT	Val (2)→Ile	MUT	+
M4S	WT	MUT	Met (4)→Ser	MUT	-

^a Encoded amino acid sequences:

VH FR1 WT : QVQLQSG;	VH FR4 WT : WGQGLTVTVSA;
V κ FR1 WT : DVVMTQTP;	VH FR4 MUT: WGQGTMTVTSS;
V κ FR1 MUT : EIVSTQSP;	V κ FR4 WT : GTKLEIK
	V κ FR4 MUT: GTKLVIK

^b hCG was coated at concentrations of 4 μ g/ml; periplasmic fractions were tested in a twenty fold dilution; -: OD₄₅₀ = 0.05 - 0.08 ; +: OD₄₅₀ > 2.00.

In conclusion, we observed that the substitution of methionine on position 4 of V κ by serine in AB3 and AB4, both having a subgroup II kappa light chain, was associated with negative ELISA results. In contrast the class IV kappa light chain containing AB2 with leucine as wild type residue tolerates the serine residue introduced by a *SalI* site. We examined the production levels of all mutants and quantified the reactivity in ELISA and with surface plasmon resonance.

4.2 Analysis of reactivity

The scFv concentration in the periplasmic fractions was determined on Western blot with the anti-FLAG antibody. The measured sample signal was compared with the signals of a range of purified scFv standards present on the same blot.

All periplasmic fractions were tested in ELISA (Table II) and surface plasmon resonance (Fig. 2) for binding with hCG. The most striking results were obtained with the mutant proteins encoded by the AB4 constructs containing serine on position 4 (clones HF4-A and M4S).

Table II. Antigen binding characteristics and FR1 sequences of the recombinant scFv's.

AB	Clone	sequence FR1-VK	Prod ^a µg/L	ELISA ^b		BIA αhCG ^c	
				OD ₄₅₀	µg/L	RU	µg/ml
AB2	WT	12345678 KIVLTQSP	355±42	0.37±0.04	178	320	1.8
	HF2	E--S----	256±59	1.45±0.10	128	870	5.1
AB4		12345678					
	WT	DVVMQTQP	462±14	2.22±0.06	116	1438	2.3
	PH1	E--L--S-	153±30	2.45±0.16	38	683	0.8
	PH2	K--L--S-	140±35	1.71±0.25	35	615	0.7
	V2I	-I-----	411±53	2.33±0.09	103	1554	2.2
	HF4-A	EI-S--S-	52.0±6.9	0.072±0.008	260	270	1.0
	M4S	---S----	15.0±4.0	0.073±0.008	75	141	0.3
AB3	WT	12345678 DVVMQTQP					
	PH1	E--L--S-	30 ^d	2.75±0.00	150	382	0.3
	HF3-A	EI-S--S-	4 ^d	0.065±0.006	20	16	0.04

^a The amount of scFv was determined on western blot using a standard curve of purified scFv. ^b For ELISA plates were coated with 4µg/ml hCG; background signals were in the 0.05 - 0.08 range, the used concentrations of scFv in ELISA are indicated. ^c Surface plasmon resonance analysis was performed with a high density coat of hCG (4369 RU) (see Fig. 1). The hCG-reactivity was determined at timepoint t=400 s. ^d Single observation.

When tested in ELISA no signals above those of the negative controls were found, however, with surface plasmon resonance we identified distinct signals. For the VK FR1 mutants residual binding was clearly detectable (clones AB4-HF4-A and AB4-M4S in Table II). Indeed, the sen-

(A)

Increased off-rate Ser4-mutant

(B)

Position 4 mutants of AB3

(C)

Compensation mutant AB4-M4S

Figure 2. (Previous page) Analysis of the scFv's on hCG binding with surface plasmon resonance (BIAcore).

- (A) Binding profiles of the AB4 derivatives with serine on position 4 (M4S: clone AB4-HF4-A) revealing a fast dissociation rate. The binding profiles of two other AB4 derivatives (M4: functional scFv from clone AB4-HF4-E; Q6E: non-functional scFv from clone AB4-Q6E) are also shown. At $t = 100$ sec the association phase started by injection of periplasmic fraction. At $t = 340$ s the surface was washed with Hepes-buffered saline (HBS) buffer.
- (B) Overlay plot of position 4 mutants of AB3. Injection was started at $t = 140$ sec, and washing at $t = 400$ sec. The serine containing scFv (M4S: clone AB3-HF3-A) also gave faster dissociation rates than the control (M4L: clone AB3-PH1 with leucine).
- (C) Improved off-rate of AB4-M4S mutant as the result of substitution of residue number 24 arginine by threonine (clone M4Smut). Overlay plot of four AB4 derivatives injected at $t = 110$ sec, washing started at $t = 240$ sec.

sensorgrams gave association profiles indicating that scFv binds to hCG, but these also revealed a faster dissociation than seen with the WT clones (compare the sensorgrams of clone AB4-HF4-E having methionine as residue number 4 with clone AB4-HF4-A with serine in Fig. 2A). It has to be emphasised, that the associations were only found when high concentrations scFv ($0.3 \mu\text{g/ml}$ or more) were injected on high density surfaces, which is an indication for a low affinity interaction. Upon dilution the signal of the serine containing clone was reduced more rapidly: from 700 to 200 RU by a twofold dilution for AB3-HF3-A with serine, contrasting the decrease from 500 to 200 RU by a fivefold dilution of AB3-PH1. In contrast to surface plasmon resonance, the bound scFv molecules are not detected in ELISA (Table II), probably as the result of extended washes. In conclusion, the M4S mutation reduces the affinity of binding in AB4. The substitution by leucine did not affect binding, as can be seen with the mutants AB4-PH1 and -PH2.

AB3, which like AB4 has a class II V κ light chain, also gave no positive signals in ELISA when methionine was replaced by serine (clone HF3-A in Table II). Again, leucine did not influence binding (clone AB3-PH1). In surface plasmon resonance binding profiles were obtained on high density surfaces only, which revealed again more enhanced dissociations than observed with the functional derivatives (Fig. 2B).

The sensitivity of the hCG ELISA should be sufficient for the detection of the scFv's expressed by the V κ position 4 mutants. For instance, AB fragments produced by the serine containing clone AB4 HF-4A gave no specific signals when tested at a concentration of 260 ng/ml, while the wild type scFv gave an OD of 2.22 even at 116 ng/ml.

By substitution of methionine with serine in the light chain of AB3 and AB4, scFv's were produced that did not react in ELISA, but in BIAcore we obtained sensorgrams, which might suggest a low affinity binding. We previously identified a mutation on position 6 of VH, that completely abolished antigen recognition. When the wild type residue glutamine of antibodies with a class IIA VH was substituted by glutamic acid, antigen binding could not be demonstrated. The position 6 VH mutants never showed such residual antigen binding with surface plasmon resonance (clone AB4-Q6E in fig. 2A) as the result of incorrect folding, what

was demonstrated by an increased sensitivity for trypsin digestion. It is interesting to examine the conformation of the antibody fragments produced by the position 4 V κ mutants in respect to their residual binding activity.

4.3 Analysis of conformation by trypsin digestion

The stability of the scFv fragments expressed by the position 4 V κ mutants was analysed by trypsin digestion as was done in other studies (Minsky *et al.*, 1986); Glockshuber *et al.*, 1992). By combining the periplasmic fractions in a single digestion mixture we could directly compare

Figure 3. Trypsin accessibility of scFv fragments of position 4 V κ and position 6 V H mutants derived from AB2 and AB4. Trypsin addition is indicated with + and -; the samples of lanes 2 and 6 in fig. A and of lanes 2 and 5 in fig. B were incubated with 0.2 mg/ml trypsin, those from lanes 3 and 7 in fig. A and in lanes 3 and 6 in fig. B with 40 mg/ml. Lane 4 (AB4-Q6E), lane 7 (AB4-M4S and AB2-WT) in fig. A, and lane 7 (AB2-Q6E and AB4-WT) are controls (no digestion).

the stability of wild type and mutant scFv's. The scFv fragments from AB2 and AB4 were separated by electrophoresis and detected on blot with anti-FLAG. The AB fragment of clone

AB4-M4S showed to be more trypsin resistant than those of clones AB4-Q6E (Fig. 3A) or AB2-Q6E (Fig. 3B), but less than the scFv's of AB2-WT (Fig. 3A) or AB4-WT (Fig. 3B). The scFv's of the Q6E mutants degraded more rapidly because these molecules do not have the protective immunoglobulin fold. The M4S mutant has an intermediate position in terms of trypsin resistance. Probably the mutant molecule adapts the immunoglobulin fold, but the mutation has a more local effect restricted to the FR1 region of V κ .

4.4 Compensating mutations for the S(4) mutant in V κ of AB4

The fact that only antigen binding of AB3 and AB4 suffered from introduction of serine at position 4, and not AB2, already indicates that this residue may generically influence the binding affinity of light chain variable domains of the kappa II family. We further studied the effect that additional mutations in the antibody scaffold might have on restoring the expression and binding of the AB4-M4S variant.

Examination of the structure of antibody AN02 directed against 2,2,6,6-tetramethyl-1-piperidinyloxy-dinitrophenyl (PDB-code 1BAF (Leahy *et al.*, 1988), aligned sequence in Fig. 1) with leucine at position 4 (Fig. 4B) showed that its neighbouring residues are the non polar alanine25 in CDR1 and threonine97 in CDR3. In contrast, the structure of a murine 4-4-20 antibody directed against 2-methyl-2,4-pentanediol (PDB-code 4FAB (Herron *et al.*, 1989), sequence in Fig. 1) with methionine on position 4 of V κ revealed serine25 and again threonine97 as neighbouring residues, as in antibodies AB4 and AB3, which are both sensitive for the M4S mutation. In AB2 the residue number 97 is the apolar alanine. Therefore we speculate that the presence of these two alanines (and thereby preventing interactions between the polar residues of positions 25 and 97 with residue 4) may make the antibody resistant to the M4S change, and we mutated serine25 and threonine97 of AB4 into alanine, in the context of the wild type methionine or the mutant serine on position 4. The versions with both M4S and T97A mutations, in combination with S25A or alone, were completely inactive as observed with surface plasmon resonance. This indicates that the threonine to alanine mutation in CDR3 predictably reduces binding. The version with M4S and S25A mutations gave an identical association and dissociation curve in surface plasmon resonance analysis as the M4S mutant. On the contrary, the wild type constructs (methionine at 4) with the alanines (single and double mutants) gave profiles similar to AB4-WT (somewhat enhanced dissociation). Thus these mutations do not affect antigen binding (despite their position in the CDR regions), but can not restore activity of the M4S variant.

A second hypothesis was based on differences in residue 24 of V κ , which is positioned nearby residue 4. In antibody 4-4-20 residue 24 is a positively charged residue (as in AB3 and AB4), while antibody AN02 and AB2 have polar residues (respectively serine and threonine) on this position. We decided to substitute arginine 24 by threonine in AB4. The S4 mutant having threonine gave a dissociation in surface plasmon resonance analysis, which is slightly better

Figure 4. (A) Presentation of the Fv structure of antibody AN02 (PDB 1BAF) made with the program Sybyl (Tripos) showing the location of residue number 4 of V κ (leucine in orange) in relation to the intradomain disulfide bond (yellow).

(B) A detailed picture of the position 4 residue of V κ (in orange, represented as Vanderwaals spheres), the disulfide bond (yellow, as Vanderwaals spheres) and its neighbouring residues serine24, alanine25 and threonine97 (in colour backbone, represented as sticks), C α -trace representation of the light (green) and heavy (yellow) chains of the Fv-domain. These images illustrate the buried position of residue 4 between the N terminus of the L chain and the amino terminal part of CDR L1 (blue) and the carboxy terminal part of CDR L3 (purple). The oxygen atoms of the side chain groups in serine24 and threonine97 are shown in red, the attached hydrogen atoms in white.

than of clone M4S (clone AB4-M4Smut in Fig. 2C), but in ELISA no clear signals were found (results not shown).

By examination of the structures of related antibodies we identified a number of neighbouring residues, which might be interacting with the mutant residue serine. However, upon grafting in the M4S mutant of AB4 the antigen binding activity, as found for the wild type constructs, could not be restored.

5. Discussion

5.1 *V κ residue number 4 affects antigen binding*

We demonstrate that the serine residue at position 4 of VL-FR1, which was introduced by cloning site *SalI*, is responsible for the absence of antigen binding in ELISA of the scFv's derived from antibody AB3 and AB4. The *SalI* restriction site was chosen on the basis of the low frequency of cutting within antibody genes (Chaudhary *et al.*, 1990). The light chain variable regions of AB3 and AB4 belong to the subgroup II kappa light chain and have methionine as wild type residue number 4. In respect to antigen binding, these antibodies do not accept serine, a residue that never occurs on this position in AB-s. However, substitution by leucine did not affect antigen binding as was already found by Benhar and colleagues (Benhar and Pastan, 1995), who showed that the mutant antibody with leucine instead of methionine obtained an increased thermal stability. In AB2, which has a class IV kappa light chain and thereby leucine as position 4 residue, the mutation to serine did not affect antigen binding.

We found a similar effect with the primer encoded position 6 residue of VH. AB-s with a class IIA VH region do not tolerate the mutation of glutamine into glutamic acid, the residue which is mainly found in class IA. However, there is a major difference: the scFv produced by the mutants with serine on position 4 of V κ reacted with hCG in surface plasmon resonance analysis although with a low affinity, actually too low for obtaining ELISA signals. Such binding profiles were never obtained with the VH position 6 mutants.

5.2 *Effect on structure*

While the position 6 VH mutants can not adapt the immunoglobulin fold necessary for binding to antigen, the folding of the position 4 V κ mutants is probably similar as for the wild type. An intermediate resistance towards trypsin digestion suggests that the introduced serine may cause a local deformation. In this way the canonical fold of L1 can be influenced. Both mutation sensitive antibodies AB3 and AB4 have a class 4 loop for L1, while the loop for AB2 is different and shows to have some similarity with class 8 (classifications were made with Chothia canonical class assignment software on internet: <http://www.biochem.ucl.ac.uk/~martin/antibodies.html>). L1 on its turn may be directly involved

in binding or indirectly by influencing the position of the other variable loops. It is remarkable to notice the high degree of homology of the V κ domains of AB4 and the 4-4-20 antibody, from which the three dimensional structure has been determined in complex with its antigen fluorescein (Davies *et al.*, 1990). Only eight residues are different: five in CDR1, one in FR2, CDR2 and in FR3 (see Fig. 1). It is interesting that the relatively long CDR1 loop of antibody 4-4-20 harbours three of the residues involved in binding to the antigen. In analogy with antibody 4-4-20 the light chain CDR1 of the two anti-hCG's AB3 and AB4 might be crucial for binding, explaining the drastic effect of mutation of the position 4 residue into serine.

Examination of the published structures of related antibodies showed that the side chain of residue number 4 is oriented towards the hydrophobic core, as is the case with the previously mentioned position 6 residue of VH. It is positioned against residues 25 and 97, that are polar in AB-s with methionine or non polar in the tolerant AB2 with leucine on 4. Exchange of polar residues in the serine sensitive AB4 by non polar residues of the serine tolerant AB2 did not improve the binding activity. In contrast, when a charged residue (arginine) on position 24 of AB4 was substituted by a polar residue (threonine), a slower dissociation was found with surface plasmon resonance than for the M4S mutant. However, the improved dissociation behaviour did not result in positive ELISA signals. In spite of a solvent orientation of arginine, it may interact with serine, thereby disturbing the local conformation.

5.3 Consequences for cloning of ABs

This study clearly showed that the VH and V κ consensus sequence specific primers can affect the binding characteristics of the encoded AB-fragments in contrast to what has been published before (Froyen *et al.*, 1995). We confirm their observations, that a number of residues tolerate primer encoded substitutions, but especially the residues on positions 2, 4 and 6 in the amino terminal part of the V-domains might be critical. The side chains of these amino acids, located in the first β strand, are oriented toward the hydrophobic core and might therefore interact with the side chains of other residues located in the core region and derived from the β strand preceding the CDR1-loop. As was published (Schulz and Schirmer, 1979) the three dimensional structure of a protein is essentially unaffected by mutations of residues, which have solvent exposed side groups, whereas a limited variation is allowed at loci containing residues with the side groups directed towards other parts of the protein. It was already noticed for in particular the residues number 2 and 4 of VL and number 2 of VH that these residues, as constituents of the Vernier zone, may adjust the CDR1 loops and thereby fine tune the fit to antigen (Foote and Winter, 1992).

The particular set of primers, used for amplification, and the application of restriction sites, may mutate residues of the Vernier Zone, and thereby can affect antigen binding of bacterially expressed scFv's or Fab's, due to the introduction of non-tolerated aminoacids in the involved

framework regions. This finding is of general importance for those scientist, who are working in the field of antibody engineering.

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CHAPTER 3

QUANTIFICATION OF SINGLE-CHAIN FV'S WITH SURFACE PLASMON RESONANCE

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Determination of active single chain antibody concentrations in crude
periplasmic fractions

*Bert Kazemier, Hans J. W. de Haard, Piet Boender, Bob van Gemen,
and Hennie R.J.M. Hoogenboom*

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1. Abstract

We have measured active single chain antibody (scFv) concentration under mass transfer limitation conditions using surface plasmon resonance on the BIAcore. For the creation of a standard curve scFv 4Dwt, derived from monoclonal antibody (mAb) 4D, directed against human chorionic gonadotropin (hCG), was purified by positive affinity chromatography. Determination of the active antibody fraction after purification was performed using anti-FLAG, reactive against a tag sequence C-terminally fused to the scFv. Two independent experiments showed that the activity remaining represented over 75% of the total amount of purified protein. Calibration curves on high density antigen surfaces showed a linear relationship between antibody concentration and binding rates. Periplasmatic fractions of six mutant scFvs, also derived from mAb 4D, revealed a clear difference in the amount of soluble active scFv expressed in the periplasm of *E. coli* compared with the total amount of antibody present, indicating the necessity of measuring active antibody concentrations. This rapid concentration determination method will be particularly useful for accurately comparing affinity constants, using antibody concentrations determined with the BIAcore, of the many different scFv fragments routinely isolated from phage display libraries.

2. Introduction

Phage display has become an increasingly important tool in biotechnology. By expressing the variable regions of antibodies (as single chain antibodies (Huston *et al.*, 1988, Bird *et al.*, 1988)) as geneIII fusion proteins on the tip of the filamentous phage M13 (McCafferty *et al.*, 1990), it is possible to select antigen binding phage, exposing the appropriate recombinant antibody, from a large pool of non-binders (Clackson *et al.*, 1991; Hoogenboom and Winter, 1992; Barbas *et al.*, 1993; Griffiths *et al.*, 1993). The method is very powerful: many different antibodies can be generated against the same antigen (Marks *et al.*, 1991; Griffiths *et al.*, 1993), making the availability of a fast and accurate affinity screening assay to identify the best antibody fragment a crucial step. The best screening assays use unpurified antibodies (crude *E. coli* supernatant of periplasmatic fractions) and are sensitive and robust. So far screening methods to determine the K_{eq} by ELISA (Friguet *et al.*, 1985) or the off-rate, using the BIAcore (Hawkins *et al.*, 1992) have been shown to be effective. To determine the affinity constant of an antibody-antigen interaction, it is essential to know the amount of scFv fragments in the crude preparation. In the present study we describe a rapid system for the determination of the amount of soluble reactive scFv, expressed in the periplasm of *E. coli*, using surface plasmon resonance (Liedberg and Lundstrom, 1993) on the BIAcore (Karlsson *et al.*, 1991) in comparison with the total amount of scFv present, estimated from immunoblots. Mab 4D, directed against hCG

(Birken *et al.*, 1990), was used as a model. Several mutants and the wild type scFv, derived from mAb 4D, were created. The influence of mutations in oligo encoded framework regions was examined on both reactivity and expression level.

2.1 Theoretical background

A mass transport limitation situation was created by immobilizing a large amount of antigen on the surface of a sensor chip (Glaser, 1993; Karlsson *et al.*, 1993; 1994). In this situation mass increase on the surface of a sensor chip becomes dependent only on antibody concentration. In BIAcore the rate of complex formation may be explained in terms of mass transfer of the analyte to the surface and in terms of analyte-ligand reaction kinetics (Eq. 1).

where A is the analyte, B is the ligand, K_M is the mass transport coefficient and k_a and k_d are the association and dissociation rate constants respectively. The mass transport coefficient is dependent on the diffusion coefficient and therefore depends on the molecular weight of the analyte. A high association constant and high amounts of immobilized ligand will cause the switch from A_{bulk} to A_{surface} to be the rate limiting step and therefore the overall reaction will be limited by mass transfer. For a standard one to one reaction between analyte and ligand the rate of complex formation can be described as:

$$d[AB]/dt = k_a[A][B] - k_d[AB] \quad (2)$$

Rewritten in terms of BIAcore response :

$$dRU/dt = k_a CR_{\text{max}} - (k_a C + k_d)RU \quad (3)$$

where RU is the detector response, C is the analyte concentration and R_{max} is the maximum amount of analyte that can be bound by the ligand (O'Shannessy *et al.*, 1993). Under mass transport limitation, on a high density surface, we can neglect the dissociation term giving a situation where the rate of complex formation is constant (Eq. 4).

$$dRU/dt = k_a CR_{\text{max}} \quad (4)$$

The initial binding rate will be independent of k_a and merely dependent on analyte concentration. From the sensorgrams of different known analyte concentrations the binding rates

can be determined and plotted against the concentration yielding a standard curve from which active concentrations of unknown samples can be calculated.

3. Materials and methods

3.1 Affinity purification of scFv 4Dwt

The wild type scFv derived from anti-hCG mAb 4D, coded 4Dwt, was purified from total cell lysate according to Wells (Tumor Biology Center, Freiburg, Germany, personal communication). Briefly, 1 litre of 2TY Amp Glu medium was inoculated (1:100) with overnight culture and grown to $OD_{600} = 0.5$ at 37°C. The cells were pelleted and resuspended in 1 litre of 2 TY Amp IPTG (1 mM final concentration). The culture was grown for another 2.5 hours at room temperature. The cells were harvested and lysed in 1/100 of the original volume of phosphate buffered saline (PBS); 8 M urea. After centrifugation the supernatant containing the denatured proteins was renatured by dialysis against PBS. Renatured protein was incubated batchwise with hCG-Sepharose (hCG, from urine of pregnant women purified on sephacryl S200 HR, coupled to NHS activated Sepharose; approximately 1.5 mg hCG/600 mg Sepharose, wet weight). After overnight binding (4°C) and extensive successive washings with PBS, 50 mM Tris/Cl pH 7.5; 0.5 M NaCl and 50 mM Tris/Cl pH 9.0; 0.5 M NaCl, the antibody was eluted with 0.1 M triethylamine and immediately neutralized by the addition of 0.5 vol 1 M Tris/Cl pH 7.0. The eluate was dialyzed against HBS (10 mM Hepes pH 7.4; 0.15 M NaCl; 3.4 mM EDTA; 0.05% P20). The amount of purified scFv was measured spectrophotometrically (assuming that 1 $OD_{280} = 0.7$ mg/ml; Marks *et al.*, 1991) and by micro-BCA (Pierce, according to the manufacturer's manual), as a double check. Purity was checked on SDS-PAGE followed by Coomassie Brilliant Blue staining.

Table 1. Oligo encoded N-terminal amino acid differences of the analyzed mutants.

clone	VH-FR1								VL-FR1							
4D21 (wt)	Q	V	Q	L	Q	Q	S	G	D	V	V	M	T	Q	T	P
4D11	-	-	-	-	-	E	-	-	-	I	-	S	-	-	-	-
4D15	-	-	-	-	-	-	-	-	-	I	-	S	-	-	-	-
4D16	N	I	-	-	E	-	-	-	K	-	-	L	-	-	S	-
4D17	-	-	-	-	-	-	-	-	E	-	-	L	-	-	S	-
4D200	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-

3.2 Sample preparation

Periplasmatic fractions of six mutant clones derived from mAb 4D, including the wild type scFv coded 4D21, were prepared as follows (Table 1 shows the different mutant sequences). Two different rounds of growth and induction were performed. After growth (50 ml scale) and induction, as described above, cells were harvested and resuspended in ice cold BBS (0.2 M borate pH 8.0; 150 mM NaCl; 1 mM EDTA). The resuspension volume was 1/100 of the original volume $\times$ OD₆₀₀, at the time of harvesting, thereby correcting for growth rate differences. After 1 h of head over head rotation (4°C) the cells were pelleted and the supernatant (periplasmatic fraction, PPF) was tested in the BIAcore.

3.3 Equipment and reagents

The BIAcore system, sensor chip CM5, surfactant P20 and amine coupling kit were purchased from Pharmacia Biosensor, Uppsala Sweden. MAb anti-FLAG (M2) was obtained from Eastman Kodak Company, New Haven, USA. The BCA protein assay reagent (micro-BCA) was obtained from Pierce, Rockford, USA. "Ultra free" microcentrifuge filter units (0.22 μ M) were purchased from Millipore, Bedford MA, USA. ECL immunoblot detection reagent was obtained from Amersham, Bucks, UK and RAM-HRP was obtained from DAKO-Patts, Glostrup, Denmark.

3.4 Immobilization

Coupling of anti-FLAG or hCG to a sensor chip CM5 was performed as previously described by Löfas and Johnsson, (1990). The carboxylated dextran matrix was activated by injection of 48 μ l 0.2 M N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC) / 0.05 M N-hydroxysuccinimide (NHS) at a flow rate of 3 μ l/min. 48 μ l anti-FLAG was injected (100 μ g/ml in 10 mM sodiumacetate pH 5.5). Using a flow rate of 5 μ l/min hCG (45 μ l at 150 μ g/ml in 10 mM sodiumacetate pH 5.0) was injected in a flow cell that was activated with 45 μ l EDC/NHS. Residual activated groups were blocked by injection of 35 μ l ethanolamine (1.0 M pH 8.5). Non-covalently bound protein was removed by a regeneration procedure (10 mM HCl; 5 μ l injection, three times) prior to injection of the samples.

3.5 Determination of the activity determination of purified scFv 4Dwt

Two concentrations of the purified scFv 4Dwt (200 μ l volumes, 200 and 300 nM) were incubated with hCG-Sepharose (the same material as used for the purification of the scFv) and 3A-Sepharose (a non-binding control containing mAb 3A, directed against hCG, coupled to the Sepharose). After overnight binding the supernatants were separated from the Sepharose using a 0.2 μ M microfuge filter. Protein concentrations were determined with micro-BCA (Pierce) and the supernatants were tested in BIAcore for their response on an anti-FLAG surface.

Residual reactivity was also tested directly in the BIAcore by injecting 20 μ l scFv 4Dwt diluted in HBS (300, 200, 150, 100, 75 and 50 nM, flow 5 μ l/min) on an anti-FLAG surface, followed by a constant hCG injection (10 μ l, 15 μ g/ml).

3.6 Concentration determination using the BIAcore

For the standard curves, 20 μ l, at a flow rate of 5 μ l/min, of the purified 4Dwt was injected (80, 60, 40, 30, 20, 10 nM, active concentrations) into a flow cell to which high levels of hCG were immobilized. Of the mutant-PPF's three dilutions (1:5, 1:10, 1:20, 20 μ l each) were injected.

3.7 Concentration determination using immunoblots

Periplasmatic fractions were run on standard reducing SDS-PAGE gels. After blotting on nitrocellulose, the filters were blocked with non-fat dry milk and incubated with anti-FLAG followed by RAM-HRP and ECL-detection.

4. Results

4.1 Determination of active scFv concentration

When analysing the kinetics of a reaction the correct analyte concentration is essential for determination of the association rate constant. The presence of small amounts of inactive antibody (especially with bivalent antibodies) can have a major influence on the determination of the affinity constant (Azimzadeh and Van Regenmortel, 1991). After purification of scFv 4Dwt the protein concentration was determined in two ways. Both OD₂₈₀ measurement and micro-

Figure 1. Overlay plot of scFv 4Dwt captured on anti-FLAG after binding to antigen (sensorgrams #3, 300 nM and #4, 200 nM) and on a non-binding control (sensorgrams #1, 300 nM and #2, 200 nM). The sample was injected at $t = 120$ s. Dissociation (buffer wash) started at $t = 360$ s. From this dissociation phase the remaining reactivity was calculated (see text for details).

BCA yielded the same concentration. SDS-PAGE followed by Coomassie Brilliant Blue staining of the purified sample showed no evidence of contaminating proteins (data not shown).

Since the high pH elution step used in purification can inactivate the scFv, it was necessary to determine the active proportion of the total amount of purified scFv 4Dwt. Two procedures were used for measuring the reactivity of scFv 4Dwt. First, 4Dwt was captured on hCG-Sepharose and on 3A-Sepharose (non binding control). Protein concentration determination of 4Dwt supernatants, pre and post adsorption to antigen, showed a residual reactivity of 79% ($\pm 4\%$) as determined with the micro-BCA. Supernatants from the binding experiments were screened on an anti-FLAG surface. The response obtained from the supernatants after overnight binding on 3A-Sepharose is referred to as the total response. The response from the supernatants after binding 4Dwt on hCG-Sepharose was obtained from non-bound inactive material. The immunoreactive fraction was determined from the dissociation phase (360-540 s) of the sensorgrams by calculating, every 5 s, the RU values from the non-bound response (Fig. 1, sensorgrams #3 and #4) relative to the RU values from the total response (Fig. 1, sensorgrams #1 and #2). RU values were determined from the dissociation phase since the response during the injection phase is influenced by the bulk refractive index of the different samples. The remaining reactivity proved to be 80% ($\pm 5\%$), which is comparable to the values obtained with micro-

Figure 2. Typical overlay plot of activity determination of scFv 4Dwt on anti-FLAG. Increasing scFv concentrations were injected at $t = 120$ s (sensorgrams: #1, 300 nM; #2, 200 nM; #3, 150 nM; #4, 100 nM; #5, 75 nM; #6, 50 nM) followed by a constant hCG injection at $t = 540$ s. RU values for calculating the immunoreactive fraction were determined at the point indicated by the arrow ($t = 670$ s).

BCA (see above). The supernatants obtained after overnight binding on hCG-Sepharose were also tested on an hCG surface in the BIAcore and showed no detectable signal indicating that all

active scFv molecules had bound to the hCG-Sepharose and that the response of this supernatant on anti-FLAG must have been associated with inactive molecules (data not shown). The responses, on anti-FLAG (Fig. 1, sensorgrams 1 and 2), of the supernatants after binding to 3A-Sepharose were the same as from the starting material (Fig. 2, sensorgrams 1 and 2) indicating that there was no non-specific binding to the 3A-Sepharose (overlay not shown).

Figure 3. Immunoblot ECL development of a typical immunoblot experiment of the second periplasmic extraction. The amount of PPF, or purified scFv, loaded on to the gel is indicated below. Lanes 1-3: 5.0/2.5/1.25 μ l 4D11; lanes 4-6: 1.0/0.5/0.25 μ l 4D15; lanes 7-9: 0.25/0.12/0.06 μ l 4D16; lanes 10-12: 0.25/0.12/0.06 μ l 4D17; lanes 13-15: 0.5/0.25/0.12 μ l 4D21; lanes 16-18: 1.0/0.5/0.25 μ l 4D200; lanes 19-23: 5/10/20/40/80 ng purified 4Dwt.

Second, 4Dwt was injected on to an anti-FLAG surface using different concentrations, followed by an hCG injection with a constant concentration (Fig. 2). The immuno reactive fraction was determined at a single time point, $t = 670$ s. At this point the responses were high and there was no contribution of the bulk refractive index to the signal. The response of 4Dwt was corrected by extrapolating the response at $t = 540$ s to $t = 670$ s, using the dissociation rate constant calculated from the time frame ($t = 500$ -540 s) just before hCG injection. On runs with different amounts of scFv, using Eq. (5), the ratio of bound hCG relative to the amount of bound scFv shows that 75% ($\pm 3\%$) of the purified 4Dwt was active.

$$\text{immunoreactive fraction} = \frac{\text{RU hCG}}{\text{RU scFv}} \times \frac{\text{MW scFv}}{\text{MW hCG}} \quad (5)$$

4.2 Concentration determination of scFv's

Total antibody concentrations were determined from immunoblots as shown in Fig. 3. Sample concentrations were estimated from known marker concentrations run on the same gel and denoted in Table 2.

For the analysis of active antibody concentration in crude extracts, purified scFv 4Dwt was used to create a standard curve. 4Dwt was injected in serial dilutions on to a sensor chip on which hCG (2850 RU) was immobilized. This surface proved to be extremely stable since over 50 runs, including efficient and complete HCl regeneration, could be performed with less than 10% loss

of surface reactivity (data not shown). Overlay plots of both the normal and the deduced ($\ln(dRU/dt)$ vs. t) sensorgrams, showing a horizontal straight line at constant binding rates, revealed

Table 2. Active antibody concentrations, calculated from the slopes of the sensorgrams, and total antibody concentrations determined by immunoblotting, of six different mutants after two independent growth/induction rounds.

Mutant	scFv concentration ($\pm$ SD nM)			
	1st periplasmic extraction		2nd periplasmic extraction	
	Total	Active	Total	Active
4D11	40 $\pm$ 20	0	50 $\pm$ 25	0
4D15	200 $\pm$ 100	44 $\pm$ 12	300 $\pm$ 200	32 $\pm$ 12
4D16	400 $\pm$ 200	255 $\pm$ 11	1000 $\pm$ 300	471 $\pm$ 20
4D17	800 $\pm$ 200	498 $\pm$ 11	2000 $\pm$ 350	777 $\pm$ 9
4D21(wt)	200 $\pm$ 130	182 $\pm$ 10	800 $\pm$ 250	329 $\pm$ 16
4D200	100 $\pm$ 50	36 $\pm$ 13	150 $\pm$ 20	34 $\pm$ 12

that the reaction is indeed mass transfer limited. It can be seen from Fig. 4 that binding rates are constant throughout the injection time. Only for the run with the highest concentration, (Fig. 4A, 80 nM), the binding rate decreases at the end of the injection phase due to the fact that too many binding sites become occupied and mass transport limitation was abolished. Calculations of the binding rates (k_s) were performed by choosing an area where the $\ln(dRU/dt)$ vs. t is constant (200–230 s), using the line fit command from the BIA-evaluation menu. The binding rates (k_s) were plotted against the concentration of 4Dwt (Fig. 4C). From this calibration curve unknown active antibody concentrations could be determined. Crude periplasmatic fractions of six mutants from two independent growth and extraction experiments were injected on the same high density hCG surface. Fig. 5 shows an overlay plot of the sensorgrams of different mutant clones. One mutant expressing non-reactive scFv (coded 4D11) shows a block signal due to a change in refractive index of the sample but no mass increase (although there is scFv present, as could be determined on the anti-FLAG-surface, data not shown, and on immunoblot, Fig. 3). Differences in the amount of active soluble periplasmic expressed scFv are clearly seen in Fig. 5. Clones 4D16 and 4D17, examples of high expression, derived from a phage library contained more active scFv than the wild type 4D21 and the other mutants. Compared with other reactive clones, mutant 4D200 and 4D15 showed evidence of a much higher off-rate. Table 2 summarizes the active scFv concentrations of the different mutants.

5. Discussion

A fast and reliable method for antibody concentration determination is of extreme importance when accurate affinity constants have to be determined, especially in the case of selection of antibodies with closely related affinities (e.g. selection of affinity matured scFvs from a phage display library, Hawkins *et al.*, 1992). Time consuming purifications should be avoided and this is where surface plasmon resonance offers a possible solution.

Figure 4. Standard curve of scFv 4Dwt on hCG. A: increasing concentrations of 4Dwt were injected onto a high density antigen surface (from top to bottom: 80, 60, 40, 30, 20 and 15 nM respectively). B: deduced sensorgrams ($\ln(dRU/dt)$ vs. t) of Fig. 3A. C: plot of the slope K_s (RU/s) as a function of the active 4Dwt concentration (nM).

In order to set up a generic method for measuring total scFv concentration, mAb anti-FLAG, directed against an eight amino acid epitope fused to the C-terminus of the scFv, was used. Over 15000 RUs of mAb anti-FLAG were immobilized on the surface of a sensor chip in order to achieve a mass transport limitation. However, from both Fig. 2 (association phase of scFv 4Dwt on anti-FLAG, $t = 120$ -360 s) and from the calculated limit coefficient, $k_a[B]/K_M$, it is clear that binding rates of scFv on the anti-FLAG are not limited by mass transport. According to Karlsson *et al.* (1994), the limit coefficient can be used to distinguish between mass transport limited and interaction controlled kinetics. For the binding of scFv 4Dwt to anti-FLAG the limit coefficient, $k_a[B]/K_M \approx 0.1$, is much smaller than 1, indicating that the reaction rate is much higher than the mass transport rate, yielding true interaction controlled kinetics.

An alternative method for measuring scFv concentration uses a high density antigen surface and requires a well known standard. During the course of this work Abraham *et al.* (1995) reported a method for measuring the approximate concentration of scFv, Fab, diabody and IgG in unpurified samples by mass transport limited binding on high density antigen surfaces on the BIAcore. One of their conclusions was that a 'gold standard' had to be created. In this study we describe the creation of the so called 'gold standard'. Two different BIAcore binding studies revealed comparable reactivity percentages of the purified scFv 4Dwt. One flaw is that for the Sepharose binding experiments very high concentrations of purified scFv are required to obtain a signal above the noise level on an anti-FLAG surface in the case of the non-reactive fraction (Fig. 1). In this case 300 nM total scFv 4Dwt containing 60 nM inactive scFv gave a response of less than 100 RU. Another drawback of this method is the difference in dissociation rate between samples with high and low analyte concentration. Samples with a high scFv concentration (3A-Sepharose supernatants) have a much higher initial dissociation rate than samples with a low scFv concentration. This can be explained by the fact that with low analyte concentration present on high density surface rebinding can occur leading to a lower dissociation rate. This difference

Figure 5. Sensorgrams of mutants and the wild type (4D21) scFvs derived from mAb 4D injected onto a high density hCG surface. The periplasmic fractions were diluted 1/5 in HBS, except clone 4D17 which was diluted 1/10 in HBS.

in dissociation rate results in a relatively high standard error. In the first part ($t = 360\text{--}420$ s) of the dissociation phase the amount of total scFv bound on anti-FLAG is higher (Fig. 1, sensorgrams #1 and #2) relative to the amount of inactive bound scFv (Fig. 1, sensorgrams #3 and #4) as compared with the end part of the dissociation phase ($t = 420\text{--}540$ s). The reactivity determination by sequential injections of scFv followed by hCG on the anti-FLAG surface on the BIAcore, as shown in Fig. 2, is preferred because of its speed, sensitivity and reproducibility.

The purified scFv 4Dwt was used to create a standard curve on a high density hCG surface. From Fig. 4C it can be concluded that there is a clear linear relationship between antibody concentration and binding rate indicating that under mass transport limitation conditions an increase of mass on the surface is only concentration dependent. Two independent experiments showed that the standard curves were identical (statistical data not shown). By using this calibration curve we were able to determine active scFv concentrations expressed in the periplasm of different mutant scFvs derived from mAb 4D (as described in Table 1) and we compared these with the total concentrations. Two independent growth and induction rounds (Table 2) show that the expression levels are highest for the phage derived clones 4D16 and 4D17, and for the wild type construct 4D21 and that the active fraction is about the same as (4D21, 1st extraction) to one third (4D17, 2nd extraction) of the total amount of scFv present. Mutant clones 4D15 and 4D200 show similar expression levels of which one third (4D200, 1st extraction) to only one tenth (4D15, 2nd extraction) is active. These clones also display a high off-rate which might be the reason why these mutants do not yield positive results in an ELISA (during washing and incubation steps the scFv is probably washed out leading to an ELISA-signal that does not exceed the background, data not shown). Mutant 4D11 contained no detectable reactive scFv although there is a clear amount of scFv present on immunoblot. Although there is some variation in the amount of active scFv produced by a single mutant in independent extractions, a clear difference in expression level of total and active scFv between different mutants can be seen.

Our conclusion is that surface plasmon resonance under mass transport limitations, by using large amounts of immobilized antigen, is a very useful and sensitive method for the rapid measurement of active single chain antibody concentration in crude extracts which avoids the need for sample purification. Comparison of active and total concentrations underline the necessity of measuring active concentrations since up to 90% of the total amount of protein present may be inactive. A drawback of the BIAcore method is that only concentrations of scFvs with high association constants, $K_a > 1 \times 10^5$ 1/Ms, can be measured. Lower K_a 's, $< 1 \times 10^5$, do not yield mass transport limited association and therefore cannot be measured using the BIAcore approach. The creation of a generic method for determining total scFv concentration, on anti-FLAG under mass transport limitation conditions, proved to be impossible. This is due to the relatively low association constant, 4×10^4 1/Ms, of the anti-FLAG for scFvs leading to the

necessity of immobilizing over 150000 RUs of anti-FLAG (which is about five-fold higher than the detection limit of the system) in order to achieve mass transport limitation situation.

6. References

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CHAPTER 4

SINGLE-CHAIN FV'S AGAINST HCG FROM A MURINE IMMUNE LIBRARY

Submitted as

A comparative molecular analysis of hybridoma-derived and combinatorial library-derived monoclonal antibodies to human Chorionic Gonadotropin

*Hans J.W. de Haard, Bert Kazemier, Cora de Jongh, Ebo Bos, Bob van Gemen,
Jan-Willem Arends, and Hennie R.J.M. Hoogenboom*

1. Abstract

In the last decades, detection of urinary human chorionic gonadotropin (hCG) has been the hall mark of early pregnancy tests. Panels of antibodies, made via hybridoma technology, exist that have solved the intrinsic complexity of this test, which are the presence in pregnancy urine of (i) high concentrations of biologically inactive fragments derived from the β -unit of hCG, and (ii) the closely related human luteinizing hormone (hLH) and human follicle stimulating hormone (hFSH). Here, we have used phage display to study the murine immune response against hCG and to isolate antibodies to many different epitopes on this antigen. Immune antibody repertoires were constructed from immunised mice and single chain antibodies (scFv's) with hCG binding were retrieved from these repertoires by biopanning or selection using labelled hCG in solution. ScFv's with most of the expected binding specificities were retrieved: (i) scFv's specific for the α -subunit, thus reacting with the gonadotropins hCG, hLH and hFSH; (ii) scFv's reactive against the β -subunit of both hCG and hLH; (iii) scFv's specific for the β -subunit of hCG; and (iv) an antibody, which only binds to the intact heterodimer of hCG and hLH. Analysis of the variable (V-) gene sequences indicate that the antibodies of the (ii) group use V-germline segments of the same V_H family as those of the hybridoma-derived monoclonal antibodies (mABs) with the same specificity, but that in the other groups no other comparable genetic homologies were found. The availability of the encoding Ab-genes in combination with phage display allows the manipulation of affinity and avidity, which might lead to the creation of molecules for a new generation of sensitive and rapid pregnancy assays.

2. Introduction

The hormone human chorionic gonadotropin (hCG) regulates steroid secretion by the corpus luteum during the early stages of pregnancy, until the placenta itself can take over this function. hCG is a member of the family of glycoprotein hormones, which also contains the structurally closely related human luteinizing hormone (hLH), follicle stimulating hormone (hFSH) and thyroid stimulating hormone (hTSH). The hormones are heterodimers sharing an identical α -chain, and an unique β -subunit that confers target-specificity. The α -chain contains 92 residues with five disulphide bridges, while the β -chain, with six disulphide bridges, varies in length from 114 residues (in hLH and hFSH) to 145 (in hCG). The recently determined structure of hCG revealed a similar conformation for both individual subunits, each having a central cysteine knot with, on one site of the knot, a loop of double stranded β -sheet-like structure, and, on the other site, two hairpin loops [1]. Despite differences in the β -chain, it was presumed that the related hormones have a similar basic structure, because all cysteine residues involved in the knot are

conserved in all members. With respect to the first 114 amino acid residues of the β -chain, hLH is more homologous (85%) to hCG than to hFSH (36%); this high degree of homology of hLH and hCG reflects their capacity to bind to the same receptor.

Measurement of hCG, intact or nicked, possibly combined with the detection of free β -subunit or the proteolytical fragments derived from the hCG β -chain [2, 3] (such as the β -core fragment (β -cf)), forms the basis of immunoassays for diagnosis of pregnancy (excellently reviewed in [4]). From the structural and sequence similarity, it will be clear that the presence of high concentrations of hLH, also found in pregnancy urine, will complicate such assay [5]. Another problem is the presence of the major break-down product of hCG, the urinary β -core fragment, which contains two polypeptide chains hCG β 6-40 disulphide bridged to hCG β 55-92; it can be found in pregnancy urine at even higher concentrations than intact hCG, but also in patients with different types of cancer [6, 7]. Fortunately, the application of certain combinations of monoclonal antibodies in a sandwich-format enables the specific detection of the intact heterodimeric hCG, which is the bioactive molecular form. Recently assays have been developed for the detection of degraded or dissociated hCG, aiming at the diagnosis of trophoblast disease, Down syndrome pregnancies, preeclampsia, and testicular and bladder cancers.

During the last decades large panels of murine monoclonal antibodies were generated for diagnostic assays, and much knowledge has been gathered on the recognised epitopes of hCG. With hybridoma technology it proved to be relatively difficult to get antibodies to hCG specific epitopes, since most are hLH cross-reactive (see for instance [5]) or recognise one of the free subunits. In this study we used phage display to obtain a series of anti-hCG antibodies. Until now, only a few reports have been dedicated to the molecular comparison between phage displayed and hybridoma derived antibodies [8], therefore combining the knowledge of sequences and specificities of the mABs with the data from the phage antibodies resulted in a detailed analysis of the murine immune response against hCG. In addition, we evaluated combinatorial library technology for the isolation of antibodies, with good characteristics (in terms of specificity and affinity) for pregnancy testing.

3. Materials and methods

3.1 Sequence analysis of α hCG mAB's

In this study four α -hCG murine monoclonal antibodies (IgG1, kappa), made by immunisation of Balb/c-mice (100 μ g hormone, purified from urine [9], was used for the primary injection, 25 μ g as boost) were examined. One group of antibodies, containing mAB 147, mAB 212 and mAB 214, recognise the β -subunit, while mAB 121 binds the holo-form of the hormone. RNA was isolated [10] from 5×10^7 hybridoma cells yielding approx. 500 μ g RNA. For the determination of the complete wild-type regions, the V-genes were amplified on random

primed cDNA, synthesised from 0.5 µg total RNA, with primers located in the constant region and the leader sequence (listed in [11]). The PCR-fragments were cloned into the T-vector (Promega, Madison, USA) and completely sequenced.

3.2 Library construction and selections

Two Balb/c mice were immunised with 100 µg hCG (same source as mentioned before) with Freund's complete adjuvant. After ten days, one mouse was sacrificed and its spleen collected. The other mouse was boosted one month after the first immunisation with 25 µg hCG in combination with incomplete adjuvant. Fourteen days later the spleen was also collected from this mouse. Total RNA was extracted from the spleens [10] (360 µg for the boosted mouse and 92 µg for the other one); 10 µg was transcribed into cDNA using random priming.

The amplification of V-genes (including the sequence of the used oligonucleotide primers), and their cloning was performed as described [12]; the IgM specific primer contains the sequence 5'-AGA CAG GGG GCT CTC GCA GGA GAC-3'. For library construction we used the phagemid vector pV1, which is identical to pHEN1 [13], except that it has the FLAG-tag [14] in stead of the *c-myc* derived tag. Transformations were performed by electroporation [15] in JM101. The ratio of insert containing clones was determined with PCR analysis as was performed by Marks and colleagues [16].

The rescue of phagemid particles was essentially performed as described [16]. For biopanning, hCG purified from urine [9], was coated in a six wells tissue culture plate (Costar, Cambridge MA., USA) at a concentration of 25 µg/ml in 50 mM Na₂CO₃ pH9.6. Selections were carried out using 10¹¹ phage particles in 1 ml of sample diluent (PBS; 20% (v/v) normal goat serum; 1.1% (v/v) triton X-100). The wells were washed and bound phage eluted with 0.1 M triethylamine (TEA) as described [16]. For selection *in solution*, hCG was biotinylated with NHS-Biotin (Pierce, Rockford, USA) at a ratio of twenty molecules Biotin to one molecule of hCG. Phage, 10¹¹ particles in 1 ml sample diluent, and 100 nM biotinylated hCG were incubated for two hours; the phage antigen complexes were captured with streptavidin-coated paramagnetic beads (Promega, Madison, USA) [17]. Further washes and the elution with TEA was performed as described [16].

3.3 Screening of individual clones and purification of scFv

Individual clones were screened for the production of hCG-reactive scFv's as described before [16]. Culture supernatants were diluted five-fold in sample diluent and tested in ELISA on plates coated with 4 µg/ml hCG in 50 mM Na₂CO₃ pH 9.6. ScFv bound to hCG was detected with mouse anti-FLAG antibody M2 (Kodak IBI, New Haven, USA) and anti-mouse-HRP conjugate (DAKO, Glostrup, Denmark). Unique clones, identified by *Bst*NI-fingerprinting, were sequenced with a thermocycling kit (Amersham, Buckinghamshire, UK) on the ALF-sequencing machine (Pharmacia, Lund, Sweden). The antibody families used in scFv-genes were determined

by the Family Assignment Software available on the Kabat Database homepage (<http://immuno.bme.nwu.edu/famgroup.html>). The most homologous germline V_H- and V_K-sequences were found in the "Germline gene directories of the mouse" homepage (http://www.ibt.unam.mx/~almagro/V_mice.html).

ScFv was affinity purified on hCG-columns, prepared by coupling of 8.4 mg hCG per gram Tressyl sepharose according to the suppliers instructions (Pierce, Rockford, USA), after refolding of the total bacterial proteins from a 50 ml culture, as was described elsewhere [18]. The yield was determined by measuring the optical density at 280 nm, assuming that a scFv has a molar extinction coefficient ($E^{1\%}_{280\text{nm}}$) of 14.3 [19]. The purity was evaluated on a Coomassie stained gel.

3.4 Analysis of specificity with ELISA, Western blot, surface plasmon resonance and pepscan

The hCG-preparation purified from urine, and immuno-affinity purified recombinant hLH, hFSH and hFSH-CTP, all produced in CHO-cells [20, 21], were used for Western blot studies as was described [22] and for ELISA. All selected scFv's were tested in ELISA using a tenfold diluted periplasmic fraction on hCG, β -cf (purified from urine according to previously described methods [23]), and hFSH (all coated at 4 $\mu\text{g/ml}$ in Na_2CO_3 pH 9.6) using the method described in section 3.3. For Western blot analysis, between 0.5 and 1 μg of each hormone was loaded per lane; the proteins were diluted in sample buffer with and without DTT, and boiled during 5 minutes or directly loaded on gel without heat-treatment. The blots were incubated during 16 hours at room temperature with 1 to 6 $\mu\text{g/ml}$ purified scFv or with 3 $\mu\text{g/ml}$ monoclonal antibody, diluted in TBST (10 mM Tris-HCl; 150 mM NaCl; 0.05% (v/v) Tween 20; pH 8.0). ScFv was detected with mouse anti-FLAG M2 and anti-mouse Ig alkaline phosphatase (AP)-conjugate (Promega, Madison, USA), and the monoclonal antibodies with anti-mouse AP-conjugate.

The specificity of the scFv's was further characterised with surface plasmon resonance on a BIAcore machine (BIAcore AB, Uppsala, Sweden) using the different glycoprotein hormones. The recombinant hLH, hFSH and hFSH-CTP were biotinylated with NHS-Biotin (Pierce, Rockford, USA) as was described before for hCG. The biotinylated hormones were immobilised on streptavidin coated chips (SA-chip, BIAcore AB, Uppsala, Sweden), yielding a surface with 1375 RU hCG, 1906 RU hLH, 1529 RU hFSH, and with 1221 RU hFSH-CTP. Purified scFv's were injected on all surfaces at concentrations varying from 12 to 99 $\mu\text{g/ml}$, while the mAb's were used at a concentration of 50 ng/ml.

Epitope mapping was performed with the pepscan method [24]. Overlapping dodecapeptides covering the entire sequence of both the α - and β -chain, coupled to the solid support, were tested with scFv. The purified scFv of clone A61 was diluted to a concentration of 3 $\mu\text{g/ml}$ in PBS containing 5% (v/v) horse serum, 5% (w/v) BSA and 1% (v/v) Tween 20. After 16 hours incubation at 4°C scFv bound to the peptides was detected with anti-FLAG M2 and anti-mouse Ig horseradish peroxidase (anti-mouse HRP) conjugate.

3.5 Affinity measurements in solution

As was recommended in the BIAevaluation software for the measurement of the “in solution” affinities, we prepared a high density coated CM-chip with hCG (4200 RUs) using the NHS/EDC-kit (Pharmacia, Lund, Sweden). A fixed amount scFv, Fab or mAB, which gave a signal between 1000 and 1500 RUs when injected on the hCG chip, was mixed with a variable amount of antigen in a volume of 60 μ l with Hepes Buffered Saline (HBS; 10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.05% (v/v) surfactant P20, pH 7.4) as dilution buffer. After an one hour period, needed for settlement of equilibrium, the samples were injected. For the quantification of free AB a standard curve was prepared by injection of a dilution series of the tested AB on the same flow cell as was described before [25].

4. Results

4.1 The murine immune response against hCG as found by hybridoma technology

During the last twenty years, large numbers of anti-hCG mABs have been made for diagnostics. In pregnancy assays it is of importance to detect the intact heterodimer, and not one of the free fragments or closely related hormones. The combination of antibodies with different specificities solves these problems, for instance capture with an α -subunit specific antibody and detection with a β -unit, hCG-specific antibody ensures the specific detection of the intact hormone of interest, being hCG. In the last decade, extensive screening of hybridomas derived from 32 fusions in our laboratories yielded a number of interesting mABs. The most frequently retrieved type of antibodies are the β -subunit, hLH cross-reactive antibodies, with mAB 212 and mAB 214 as representatives. Another often found specificity was directed against the α -subunit, here represented by mAB 116. Only two mABs, which recognised the intact heterodimer of hCG and hLH, were isolated; we chose mAB 121 from this group for further analysis. It turned out to be extremely difficult to select hCG-specific antibodies: from all fusions a single mAB was obtained, that recognised the carboxy terminal peptide of the β -unit of hCG (β -CTP; nomenclature in [26]); it was coded mAB 293.

For studying the immune response against hCG on a molecular level, we used phage library technology. First of all, we were interested to see whether the specificities of the selected recombinant antibodies would match with the hybridoma derived antibodies, and secondly, if the sequences of both types of antibodies would be related. Therefore the sequences of the V-regions were determined from mABs 121, 147, 212 and 214. Their potential application in diagnostics was evaluated by analysis of specificity and affinity. The next paragraphs will discuss the selection and characterisation of the recombinant antibodies.

4.2 Construction of murine libraries and selection of anti-hCG phage antibodies

For the generation of combinatorial libraries, we immunised two Balb/c mice, the strain originally used for the generation of the hybridomas, with 100 µg purified hCG. Different immunisation schemes were applied to investigate the effect of maturation on the affinity and the specificity of the selected antibodies. One mouse (#1) was sacrificed 10 days after a single immunisation; its spleen was removed and used for isolation of total RNA. The other mouse (#2) was boosted after one month with 25 µg hCG and sacrificed a fortnight later (this was the standard protocol for the generation of hybridomas). Random primed cDNA was synthesised and applied as template for the amplification of IgG or IgM specific variable heavy chain fragments (V_H) for mouse #1, and IgG specific V_H -regions only for mouse #2, and kappa light chain region (V_K) fragments from both mice. The oligonucleotide primers used for amplification were designed on the basis of the encoding amino acid sequences of published mouse antibodies. The V_H - and V_K - encoding DNA-segments were coupled by splicing by overlap extension, as was described before [8], and cloned in phagemid pV1. Two libraries were made for mouse #1, encoded M1 containing the IgM derived V_H -regions with 5×10^6 clones (with 70% of the clones containing a scFv encoding insert), and library G1 from the IgG derived V_H fragments with 1.4×10^7 clones (the fraction of clones, which have an insert, was 85%). From mouse #2 only an IgG-derived library was generated, encoded G2, containing 1.1×10^7 clones (85% of the clones had insert).

The rescued phage of library G1 and M1 were selected separately on hCG coated onto a polystyrene surface [16]. After three rounds of panning a 200-fold enrichment was found in the number of eluted phage relative to the numbers eluted during round 1. The number of scFv expressing clones increased from 50% after round 1 to more than 95% after round 3, as was judged on morphology of the clones [12]. Among the antigen reactive clones of library G1, six different fingerprints were identified. Representative clones were taken from each pattern, which were coded A11, A61, A64, A124, C11 and C35. From the IgM library M1 we retrieved two different hCG reactive clones: A6 and A7. The mode of antigen presentation during selection was examined by repeating the selection on library G1, but instead of immobilised antigen we now used soluble biotinylated hCG (100 nM at each round). The phage titre upon elution increased in round 4 only, with an unexpected 100-fold decrease in round 2. Three hCG binding clones were identified by fingerprint analysis, i.e. clones A1, A5 and A9. Finally, the IgG based library G2, made from the spleen of mouse #2, was used for selection with soluble antigen, yielding a similar enrichment as was seen with library G1 and M1. Four antigen specific clones, coded B3, B4, B6 and B7, were identified.

In the following paragraphs we will discuss the characterisation of our panel of in total 15 different anti-hCG single chain antibodies. The specificity of the selected antibody fragments was determined with Western blot, surface plasmon resonance, and in some cases with pepscan,

revealing four classes of binding specificities. To complete the comparison with mABs, the affinities were determined with refolded, affinity purified scFvs. Since the applied refolding procedure only yields a fraction of functionally active antibody fragments, the inactive molecules were removed by affinity chromatography on hCG columns. By doing this, overestimation of the protein concentration was avoided by the presence of inactive scFvs, which would lead to lower affinities. Instead of measuring the kinetics of binding, we used the "in solution" method for determination of the affinity [27], which permits the direct comparison between scFvs, intact mABs and Fabs. The principle relies on the measurement of the amount of free analyte (AB or Ag) in a pre-established equilibrium mixture. We determined the amount of free antibody in the BIAcore machine according to the method described by Kazemier and colleagues [25]. By plotting the concentration of free AB or the fraction AB_{free}/AB_0 , in which AB_0 is the concentration AB when no Ag has been added, against the total concentration Ag, the K_d can be deduced.

4.3 Anti- α -chain scFv's clones A6, A7, B6 and B7

Western blot analysis, using the recombinant antibody as the detection probe, revealed that the specificity of clone B6 scFv is directed against the α -chain of hCG, hLH, hFSH and hFSH-CTP; the latter chimera contains the carboxyterminal peptide of the β -chain of hCG fused to the β -chain of FSH as described [28]. Although clone B6 was selected with biotinylated hCG from library G2, it recognises all hormone derivatives in both native and denatured form (Fig. 1B). When the gonadotropins were analysed without heat treatment, the intact heterodimer was detected by B6 scFv (marked with α/β in Fig. 1B). This specificity pattern is identical to that of the hybridoma produced monoclonal antibody 116 (coded mAB 6 in Fig. 1C).

BIAcore analysis with the different gonadotropins immobilised on sensorchips confirmed the anti- α -chain specificity of the scFv observed with Western blot analysis (Fig. 2B). A good response was seen against hFSH (also in ELISA), and against hLH in the sensorgrams (270 RU and 150 RU respectively immediately after injection at $t = 260$ sec in Fig. 2B). On hCG a much lower signal (approx. 50 RU) was obtained in spite of a similar antigen density on the chip; this is probably caused by the presence of free β subunit in the urinary hCG preparation used for immobilisation. Similar responses were obtained with the α subunit specific mAB 116 (420 RU for hFSH and 240 RU for hLH at $t = 260$ sec in Fig. 2C), including the relative low response on hCG (30 RU). Clones B7, A6 and A7 gave identical sensorgrams as clone B6 (data not shown), suggesting that these also recognised the α -chain. In Fig. 4 the affinity curves derived from several anti-hCG mABs and scFvs are shown; Table 2 gives the corresponding K_d -values. The affinity of the antibody fragment expressed by clone B6 was 35 nM (Table 2), which is slightly lower than of the anti- β -CTP specific mAB 293 or mAB/scFv 212 and somewhat better than of mAB/scFv 214; the two last mABs are reactive against the β -subunit.

Figure 1 (on previous page). Analysis of the specificities of the scFvs and mABs on Western blot. The gonadotropins were dissolved in non-reducing sample buffer, and boiled (T+) or not heat-treated (T-). ScFvs A64 and B6 also recognised protein, which was denatured with DDT (blots in the middle).

Panel A. Blots incubated with anti- β -subunit scFvs, of the hLH cross-reactive clone A64 on the left, and on the right hand site of the hCG specific clone A124.

Panel B. As in (A), but the left blot was probed with the α -chain specific clone B6, and the one on the right with the α/β -heterodimer recognising scFv from B3.

Panel C. Blots incubated with mABs; on the left with the anti- β -subunit mAB 147 (coded mAB 7), in the middle with the α -chain specific mAB 116 (coded mAB 6), and the one on the right with the anti- α/β -heterodimer recognising mAB 121 (coded mAB 1).

Sequence analysis (Table 1) revealed that all α -unit specific clones A6, A7, B6 and B7 contain a subgroup V heavy chain and a subgroup XVII kappa light chain variable region, except clone A7, which uses a kappa family IX. A major variability is seen in the length of the heavy chain CDR3. The closest matches with germline sequences were found with the heavy chain V-region C57G26 and the light chain V-region K2 [29]).

4.4 Anti- α/β -chain scFv: clone B3

The scFv derived from clone B3, selected from the library G2 with biotinylated hCG, recognises the intact heterodimers of hCG and hLH as determined in Western blot (Fig. 1B). The individual subunits were not detected, nor the intact heterodimer of hFSH and hFSH-CTP, confirmed by the absence of response in ELISA against hFSH and β -cf. Apparently the recognised sequence is a conformational epitope made up of both subunits. A similar reactivity was found with mAB 121 (mAB 1 in Fig. 1C), although this antibody was shown to have some cross-reactivity with the α -chain products of hFSH-CTP and hLH.

The results were confirmed in BIAcore (Fig. 2B), although the responses on hCG and hLH were rather low (40 and 80 RU respectively) for the scFv from clone B3. However, signals are comparable to the signals obtained from mAB 121 (200 and 400 RU on hCG and hLH respectively), taking into account the five-fold difference in molecular weight between an intact IgG and a scFv (see Fig. 2). As was already observed with Western blot, mAB 121 gave some degree of reactivity (50 RU) on hFSH, whereas the scFv did not have the cross-reactivity.

Table 1 (on next page). Alignment of the amino acid sequences of the variable heavy (A) and light (B) chain regions of the anti-hCG scFv-s, grouped upon their specificities, with their germline analogues.

a) On the bottom line of each group of library derived antibodies, the germline derivative is shown. The Genbank-codes and accession number of the germline V_H -regions are: for V11 MUSIGHVP and J00525 respectively, for C57G26 MUSC57G26V and L33937, for GLvh50 MUSIGHCVA and L17134, and for 37A11 MUSIGHVAD and M18951. For the germline V_L -regions these are: for Ars MUSIGKCMN and M15520 respectively, for 24B MUSIGKVV and K02418, for K2 MUSIGKVA and J00545, for 21A MMVK21A and X16954, and finally for 21C MUSIGKVR3 and K02161;

b) Library, from which the clone was selected;

b) Numbering of the residues and classification of the heavy and light chains was performed according to Kabat et al. (1991). Differences from the first member of the group are indicated with uppercase characters, primer encoded differences with lowercase characters;

a) The specificity of the scFv's are indicated with a (α -unit) or b (β -unit);

a) The number of somatically mutated germline residues are also shown (#mut), the mutated residues themselves are indicated with boxes.

A. Heavy chain

AB ^(a)	lib ^(b)	class ^(c)	spec ^(d)	#mut ^(e)	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
					10 20 30	5A	40	50 2ABC 60	70 80 2ABC 90	100ABCDEF 2	110
B3	G2	X	a/b	6	EVQLVESGGGLVQPGGSLRLSC	DYMS	WVRQSPGKAFWLG	FIRDKANGYTYE	RPTISRDNSQSILYLQMTLRAEDSATYYCAR	SYVGRYF	DV WGAGTPTVTSS
B4	G2	X	b	8	EVQLVESGGGLVQPGGSLRLSC	---	WVRQSPGKAFWLG	---	---	---	---
V11					--k--	---	---	---	---	A--RYM	--Y--Q--m--
B6	G2	V	a	8	QVQLQQSGPELVKPGASVKISCKASG	GYV	WVRQSPGKAFWLG	EINP	KATLTVDKSSSTAYMOLKSLTSEESAVVYCTR	DDNSGYF	DV WGAGTTVTSS
B7	G2	V	a	15	QVQLQQSGPELVKPGASVKISCKASG	---	WVRQSPGKAFWLG	---	---	---	---
A6	M1	V	a	6	QVQLQQSGPELVKPGASVKISCKASG	---	WVRQSPGKAFWLG	---	---	---	---
A7	M1	V	a	6	QVQLQQSGPELVKPGASVKISCKASG	---	WVRQSPGKAFWLG	---	---	---	---
C57G26					---	---	---	---	---	---	---
C35	G1	V	b	9	EVQLQESGPELVKPGASVKISCKASGYTPT	DYV	WVRQSHGKSLEWIG	YIYP	KATLTVDKSSSTAYMOLKSLTSEESAVVYCAR	EGLIRNYAM	DY WGQGLTLVTSS
A9	G1	V	b	10	EVQLQESGPELVKPGASVKISCKASGYTPT	---	WVRQSHGKSLEWIG	---	---	---	---
A124	G1	V	b	10	EVQLQESGPELVKPGASVKISCKASGYTPT	---	WVRQSHGKSLEWIG	---	---	---	---
GLvh50					---	---	---	---	---	---	---
A64	G1	VII	b	3	EVQVQQSGAELMKPGASVKISCKATGYTFS	SYWIE	WVRQSPGKAFWLG	---	---	---	---
A61	G1	VII	b	4	EVQVQQSGAELMKPGASVKISCKATGYTFS	---	WVRQSPGKAFWLG	---	---	---	---
A11	G1	VII	b	6	EVQVQQSGAELMKPGASVKISCKATGYTFS	---	WVRQSPGKAFWLG	---	---	---	---
A1	G1	VII	b	10	EVQVQQSGAELMKPGASVKISCKATGYTFS	---	WVRQSPGKAFWLG	---	---	---	---
A5	G1	VII	b	14	EVQVQQSGAELMKPGASVKISCKATGYTFS	---	WVRQSPGKAFWLG	---	---	---	---
C11	G1	VII	b	4	EVQVQQSGAELMKPGASVKISCKATGYTFS	---	WVRQSPGKAFWLG	---	---	---	---
37A11					---	---	---	---	---	---	---

B. Light chain

AB ^(a)	lib ^(b)	class ^(c)	spec ^(d)	#mut ^(e)	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
					1020	7ABCDE30	40	50	607080	905A100	
B3	G2	XIII	a/b	8	DTKMTQTSSSSASLGKRVSLT	RASQ	DIHLYN	WYQKPGDGTVKLLIY	YTSRLHS	GVPSRFSGSGSGTDYSLTISNLEQEDIATYFC	QQGNLTLP RT PGGGTKLELKR
Ars					-iq--T-L--D-TIS-	---	---	---	---	---	---
B4	G2	VII	b	2	DIYMTQNAFNSFVTLGTSASISC	RSSKSLHNSGITYL	WYLOKPGQSPOLLIY	QMSNLAS	GVPDFRSSSGSGTDYSLTISRVEAEDVGYYVC	AQNLELP T PGGGTKLELKR	
24B					-a	---	---	---	---	---	---
B6	G2	XVII	a	4	DIKMTQSPASLSASVGTETITC	RASG	NIHNYLA	WYQKQKQSPOLLYY	NAKTLAD	GVPSRFSGSGSGTDYSLKINSIQPEDFGSYYC	QHLELTP WT PGGGTKLEIKR
B7	G2	XVII	a	7	-q--t	-S-	-S-	---	---	---	---
A6	M1	XVII	a	12	-q--t	-S-	-S-	---	---	---	---
K2					-q--t	-S-	-S-	---	---	---	---
A9	G1	IX	b	10	ENVLTQSPASLAVSLGQRATISC	RASEVDYSGNSPMH	WYQKPGQPPKLLIY	RASNLES	GVPARFSGSGSGTDYSLNIHPMEEDDTAMYFC	QQSKEVP WT PGGGTKLEIKR	
21A					d1	R--E--N Y-I-F--	-F-	A--QG-	---	---	---
A64	G1	IX	b	0	EIVLTQSPASLAVSLGQRATISC	RASEVDS YGNFPMH	WYQKPGQPPKLLIY	RASNLES	GIPARFSGSGSRTDFTLTINPVEADDVATYYC	QQSNEDP WT PGGRTKLELKR	
A124	G1	IX	b	0	-f-	---	---	---	---	---	---
A61/A11/C35	G1	IX	b	0	-nf-	---	---	---	---	---	---
A1	G1	IX	b	1	d--g	---	---	---	---	-S-	R--G--I--
A5	G1	IX	b	0	-f-	---	---	---	---	---	Y--G--I--
C11	G1	IX	b	1	---	---	---	---	---	-S-	--G--I--
A7	M1	IX	a	1	-f-	---	---	---	---	-S-	R--G--I--
21C					d	---	---	---	---	---	---

The affinity of the antibody fragment produced by clone B3 (Fig. 4A), is lower than found for the mABs or scFv B6: 80 nM (Table 2). A comparison with mAB 121 could not be made, since its affinity was not determined.

Alignment of the heavy chain sequence of clone B3 revealed the use of a subgroup X variable region, derived from the germline sequence coded V11 [30] (Table 1). The scFv of clone B4, reactive against the β -subunit of hCG and hLH (discussed below), contains a V_H homologous to the one of clone B3, but its CDR3 is different in length and sequence, while the light chain variable regions of both clones are completely different (clone B4 uses a class VII kappa light chain derived from the germline 24B [31]. Clone B3 has a kappa variable light chain variable region of class XIII, derived from the germline analogue Ars [32].

4.5 hLH cross-reactive antibody fragments recognising the β -subunit of hCG

The majority of clones selected from library G1, either via panning (clones A61, A64, A11, and C11) or with biotinylated antigen (clones A1 and A5), were reactive with the β -subunit of urinary hCG. In ELISA the fragments gave positive responses with hCG and its degradation product β -cf. Clone A64 was chosen for detection of the hormones on Western blot: a clear reactivity was obtained against the β -unit (MW 36 kDa) of hCG and hLH, in non-denatured and denatured form (Fig. 1A). In the hCG-preparation purified from urine a product derived from the β -subunit (MW 28 kDa) was detected by the scFv's in non-denatured but also in denatured form, suggesting that it can not be β -core (β -cf) or the carboxyterminal part of nicked hCG (residue 48 to 145; for discussion of fragments see [4, 33, 34]). When the hCG preparation was analysed on gel, non-reduced and without heat treatment, the low molecular product could not be detected by β -unit recognising antibodies, suggesting that it is not a free fragment. Apart from free β -subunit, the antibodies recognised a molecule on blot with a somewhat lower molecular weight (40-42 kDa) as the intact heterodimer (47 kDa). We therefore believe, that the molecule represents the complete β -unit with an altered glycosylation pattern, previously described as the β -unit of the small form of hCG (SP-hCG β) [35], with shorter carbohydrate chains, rather than one of the known degradation products.

Figure 2 (on next page). Binding analysis of the scFv's directed against hCG using surface plasmon resonance (BIAcore).

- A. Analysis of the anti- β -subunit recognising recombinant antibodies. On the left the overlay plot of the hLH cross-reactive scFv from clone A5 on hCG, hFSH, and hLH is shown. From $t = 140$ sec to $t = 260$ sec the purified fragment was injected, subsequently the surface was washed with Hepes-buffered saline (HBS) buffer. On the left panel the overlay plot of the hCG specific scFv produced by clone A124 is shown.
- B. Sensorgrams of the anti- α -chain recognising scFv from clone B6 (left), and of the anti- α/β -heterodimer scFv from clone B3 (right).
- C. For comparison sensorgrams were made from the anti- β -chain mAB 147 (left), the anti- α -chain reactive mAB 116 (right), and the anti- α/β mAB 121 (below).

(A) anti- β -core

(B) anti- α

anti- α/β

(C) anti- β -core

anti- α

anti- α/β

In BIAcore a nearly identical response was found with hCG and hLH (see signals obtained by injection of scFv from the representative clone A5 in Fig. 2A). This kind of reactivity resembles the pattern obtained from mAB 147, which is also β -unit specific and hLH cross-reactive: identical responses were found in surface plasmon resonance with this monoclonal antibody on both hormones (Fig. 2C), while on Western blot it detected the β -unit of both hCG and hLH (coded mAB 7 in Fig. 1C).

Since the hormone was recognised in denatured form, we examined the epitope recognition of the representative clone A61 in more detail by pepscan analysis [24] with overlapping dodecapeptides, that cover the entire sequence of both hCG chains. A main peak can be identified in the aminoterminal part of the β -unit (Fig. 3). The sequence of the first aminoterminal peptide is SKEPLRPCRPI, and the carboxyterminal peptide of the series of eight reacting peptides contains the sequence RCRPINATLAVE, thereby indicating that the epitope recognised by A61-scFv includes the sequence RCRPI, which is also present in the aminoterminal part of the β -core fragment. Another lower peak can be discriminated around peptide number 65, but the irregular pattern suggests non-specific binding.

Figure 3. Pepscan results using the β -chain specific scFv expressed by clone A61. The analysis was performed with the overlapping dodecapeptides derived from the β -subunit of hCG, starting with the peptide number 1 with the sequence SKEPLRPCRPI and ending with peptide 134 with the sequence LPGSDTPILPQ.

The affinities from a limited number of antibody fragments were measured. The A64-scFv has an affinity of 20 nM (Fig. 4A) and the very homologous A61 35 nM (Table 2; for discussion of sequences see next paragraph). ScFv A1 has an approximately ten-fold lower affinity (300 nM; see Fig. 4A and Table 2), but coinciding with this difference in affinity some deviations in

sequence can be observed within FR1 of V_H and CDR3 of V_L when compared with clone A64 (discussed in next paragraph).

All anti- β -unit antibodies have a subgroup VII heavy chain variable region, with some variability in FR1- and CDR1-region (Table 1). Up to 8 differences (not primer encoded) in 30 residues for FR1 and 3 differences in 5 residues for CDR1 were found for clone A5, and 6 in FR1 and 1 in CDR1 for the previously mentioned A1 in comparison with the V_H germline segment encoded 37A11 [36]. The only type of kappa light chain variable region found in these antibodies was of subgroup IX, derived from the germline analogue 21C [37]. Considering the sequence of V_K, A1 was again the most divergent clone with two differences in CDR3.

One of the three clones from library G2, clone B4-scFv, recognised also the β -chain of hCG and hLH. In ELISA an indeterminate signal was found with β -cf. Its reactivity pattern, as observed on Western blot and in BIAcore (data not shown), was identical to what was found with the clones derived from library G1. Its heavy and light chain sequence completely differs from the G1 derived, β -subunit recognising antibodies (Table 1), suggesting that the epitopes involved might be different.

4.6 Reactivity against β -unit and hCG-specific: clones A9, A124 and C35

Phage antibodies specific for the β -chain of hCG were selected from library G1 using both selection methods (clones A9, A124 and C35). In ELISA positive signals were obtained with hCG and β -cf, but not with hFSH. On Western blot no cross reaction with hLH was seen, as is shown for the representative clone A124 (Fig. 1A). As a control the hLH cross-reactive scFv A5 was used; this clone gave stronger signals against hLH than against hCG on blot, while the hLH-reactivity was completely missing for A124 (compare the blot incubated with hLH cross reactive scFv A64 on the left hand with the blot on the right incubated with hCG specific scFv A124 in Fig. 1A). Similar patterns were obtained for the other clones.

This unique hCG-specificity was confirmed by surface plasmon resonance analysis. Injection of the purified scFv's derived from these clones on the hCG surface resulted in high responses, which were comparable to those found for the hLH-cross reactive antibodies, but on hLH, hFSH, and hFSH-CTP no binding could be detected (see Fig. 2A: the left sensorgram shows the binding of 550 RU scFv at $t = 260$ sec on the hCG- and hLH-surface for the cross-reactive clone A5, while an identical amount of scFv A124 was captured only by hCG, as is shown in the sensorgram of the right panel).

Within the group of the hCG-specific scFv's, a big difference in affinity was found; the affinity of scFv A9 was rather low (330 nM), while the other two antibody fragments had a ten-fold higher affinity (30 nM and 45 nM for C35 and A124 respectively), comparable to the values found for the hLH cross-reactive or the α -chain specific scFv's.

The hCG specific clones used a subgroup V heavy chain, derived from the germline sequence coded GLvh50 [38] (Table 1). Although all clones contained a class IX kappa light

chain, the germline sequence for clone A9 (coded 21A [39]) is different from clones A124 and C35: these clones use, like the previously described hLH cross-reactive clones, the germline

(A)

(B)

Figure 4. Affinity plots from scFv's (panel A) and mABs (panel B). The percentage of free AB was plotted against the antigen concentration (logarithmic scale). The amount of free AB (ie. not bound to soluble Ag) was determined on BIAcore and related to the input amount AB (no Ag added). The K_d was derived from the plots at 50% binding.

sequence encoded 21C. The ten-fold lower affinity found for clone A9, relative to the two other hCG specific clones, must be caused by the use of a different light chain germline sequence.

5. Discussion

5.1 Molecular analysis of the murine antibody response against hCG

For the evaluation of the immune response against hCG, we studied the early immune response by injection of mouse #1 with a single dose of antigen, while the effect of maturation was examined with mouse #2 by an additional boost with a decreased amount of antigen. From the libraries, constructed by PCR-cloning of the IgM-derived V_H -regions from the spleen of mouse #1 (coded library M1), and of the IgG-derived V_H -regions from mouse #1 and #2 (coded library G1 and G2 respectively), a spectrum of antibodies with different specificities was selected with intact hCG (Table 2). From the G1-library a group of β -cf specific, hLH cross-reactive antibodies were retrieved, and three β -hCG-specific antibodies (also β -cf specific), while the M1 repertoire, made from the same mouse, yielded only anti α -unit antibodies. The G2-repertoire, constructed from mouse #2, gave an even "more" diverse set of specificities, including a different β -unit specific, hLH cross-reactive antibody, two α -unit specific AB's, and one hLH cross-reactive antibody, recognising the holo-form of the hormone.

Examination of the sequences (Table 1) shows a correlation between specificity of the clones and the subgroup of the heavy chain variable region, and to some degree with the length of the V_H CDR3 (Table 1). This would imply that the heavy chain variable region would dominate the specificity, a phenomenon observed before [40]. All anti β -unit scFv's are paired with only one family of light chain V-regions, which might be an indication for maintenance of the pairing originally present in the B-cell produced antibodies [41]. This phenomenon was also found in the group of α -unit specific scFv's: all, except A7, pair with a family XVII light chain, even though the antibodies originate from different mice. Within the group of hCG-specific antibodies, one of the clones (A9) uses another (although related) light chain variable region derived from a different germline sequence (21A) as the other group members C35 and A124 (derived from germline analogue 21C). The specificity of clone A9 was not changed, but its affinity was completely altered by the use of the other V_L : the scFv produced by this clone showed to have a ten-fold lower affinity. Apparently the V_L aids in the formation of optimal interactions with the antigen and thereby influences the strength of binding; this principle actually forms the basis for light chain shuffling [42] for improving the affinity without changing the specificity.

Comparison of the sequences of the β -unit, hLH cross-reactive scFv's selected from library G1 with those from our panel of monoclonal antibodies (Table 1) shows a remarkable resemblance in the used V_H -family: both the library derived as well as the hybridoma produced,

β -unit specific antibodies (mAB's 147, 212 and 214) use V_H family VII, although originating from different, but closely related germline genes (37A11 and VH105 for the library and hybridoma derived antibodies respectively). The light chain found in the β -unit specific scFv's is closely related to the one from mAB 147: the germline genes used are 21C for the library

Table 2. Characteristics of the hCG reactive scFvs selected from libraries and hybridoma produced mABs.

Format	Name	Library	Selection method		β -chain		β -core	α/β -chain	α -chain
Ab			Soluble Ag	Panning	CG	LH		(CG/LH)	
scFv	A1	G1	X		+	+	+	-	-
	A5	G1	X		+	+	+	-	-
	A9	G1	X		+	-	+	-	-
	A11	G1		X	+	+	+	-	-
	A61	G1		X	+	+	+	-	-
	A64	G1		X	+	+	+	-	-
	C11	G1		X	+	+	+	-	-
	A124	G1		X	+	-	+	-	-
	C35	G1		X	+	-	+	-	-
	A6	M1		X	-	-	-	-	+
	A7	M1		X	-	-	-	-	+
	B3	G2	X		-	-	-	+	-
	B4	G2	X		+	+	?	-	-
	B6	G2	X		-	-	-	-	+
	B7	G2	X		-	-	-	-	+
mAB	212				+	+	+	-	-
scFv	212				+	+	+	-	-
mAB	214				+	+	+	-	-
scFv	214				+	+	+	-	-
mAB	293				+	-	-	-	-
Fab	293				+	-	-	-	-

derived antibody and 21A for the hybridoma derived antibody, and the length of the CDR1 is identical also. ScFv B4 with a V_H family X, derived from library G2, might recognise a different epitope on the β -subunit as the V_H family VII containing scFv's, selected from library G1.

The above described analogy between library and hybridoma derived anti β -cf antibodies in respect to the used heavy chain V-region was not found for the α/β -heterodimer specific ABs: scFv B3 and mAB 121 use different V_H -families.

5.2 Analysis of somatic mutations

The best case for studying the effect of maturation is formed by the group of α -unit specific antibodies, the only group of antibodies selected from the M1 library, representative for the early immune response, and from the G2-library, constructed from the boosted mouse and hence illustrative for the late response. The alignment of the heavy chain amino acid sequences (Table

1) with the germline analogue C57G26 reveals a high number of somatic mutations in the library G2 derived clone B7, especially when compared with the IgM-derived clones of mouse #1. The IgM derived clones A6 and A7 both contain 6 mutated amino acid residues, whereas clone B7 has 15 mutated residues within a stretch of 84 amino acids. As was suggested in literature [43, 44], analysis of the nucleotide sequence should give additional information, since an increased ratio of replacement compared to silent mutations is typical for somatically mutated CDRs. Apparently FRs do not tolerate most replacement mutations, as a consequence of structural constraints. Indeed, clone B7 contains as many as 32 mutations in its heavy chain V-region with most replacement mutations in its CDR1 and CDR2 (3 and 7 mutated nucleotides, see Table 3).

Table 3. Distribution of codons with replacement and silent mutations in VH- and VL-genes of library derived and hybridoma produced antibodies.

library specificity				Ratio Replacement vs. Silent Mutations (R/S)						total # mut
				FR1	CDR1	FR2	CDR2	FR3	CDR3	
scFv B3	G2	α/β	VH	1/2	0/1	2/0	3/1	0/1	X	11
			VL	7/0	5/1	0/0	0/0	0/0	0/0	13
scFv B6	G2	α	VH	1/1	1/0	3/0	3/1	0/2	X	12
			VL	0/1	0/1	0/0	0/0	1/3	5/0	11
scFv B7	G2	α	VH	5/3	3/2	5/2	7/0	2/3	X	32
			VL	0/1	3/0	0/0	1/0	1/3	5/0	14
scFv A6	M1	α	VH	1/0	1/0	2/1	2/2	1/1	X	11
			VL	2/1	3/0	0/0	3/0	3/1	5/0	18
scFv A7	M1	α	VH	0/0	1/0	1/1	4/2	0/3	X	12
			VL	0/0	0/0	0/0	0/0	0/0	1/0	1
scFv A9	G1	β -CG	VH	2/2	2/0	0/0	4/1	2/0	X	13
			VL	0/0	8/1	1/0	4/1	0/0	0/1	16
scFv C11	G1	β -CG/LH	VH	0/2	0/0	0/0	1/0	3/1	X	7
			VL	0/0	0/0	0/0	0/0	0/1	1/1	3
scFv B4	G2	β -CG/LH	VH	1/1	0/0	3/0	3/0	2/0	X	10
			VL	0/0	0/0	0/0	1/1	1/2	0/0	5
mAB 147		β -CG/LH	VH	4/3	4/0	1/1	11/1	4/2	X	31
			VL	1/1	2/1	0/0	0/0	0/1	1/1	8
mAB 212		β -CG/LH	VH	2/6	7/1	1/1	8/4	3/1	X	34
			VL	4/2	3/1	1/0	0/0	2/1	5/2	21
mAB 121		α/β	VH	1/1	0/0	0/0	0/0	0/0	X	2
			VL	0/1	0/0	0/0	0/0	1/1	0/0	3

For comparison, the IgM derived clone A6 carries 11 mutations with one and two replacement mutations in CDR1 and CDR2 respectively, and clone A7 has 12 mutated nucleotides with one and four replacement mutations for CDR1 and CDR2 respectively. By an additional immunisation with a low amount of hCG, the process of somatic mutation and antigen selection seems to be focused on CDR1 and 2. Although scFv B6 has a high affinity (35 nM), we do not know if the mutations of the V_H CDR1- and CDR2-residues really contribute to an improvement of the affinity, because those from scFv's A6 and A7 were not determined. Our data confirm the rule of the increased ratio replacement to silent mutations for the CDRs, both for phage library derived antibodies as well as hybridoma derived antibodies (Table 3). The light chain regions of antibodies from both sources have less mutations (in nucleotide and amino acid sequence) as the heavy chain regions, with some exceptions.

A comparison between antibodies selected from the G1-library with those from the G2-library, and with different specificities, does not seem to be useful as can be concluded by examination of the hybridoma derived antibodies. MAB 121, a representative for the late response, has an almost completely germline V_H- and V_L-region, whereas the anti β -cf specific mABs contain between 14 and 19 mutated amino acid residues within their V_H-region and between three and 12 mutated residues within their V_L-region. A comparable low level of mutations were found in some of the high affinity clones (A64 and A61) within the group of the hCG/hLH cross-reactive antibodies (three and four residues respectively). This is consistent with reports on the isolation of high affinity antibodies, just four days after exposure to antigen [45], and with the finding of high affinity antibodies containing germline sequences [46]. Within the group of the hCG/hLH cross-reactive antibodies, the V_H-region of one low affinity antibody (A1) seems to be heavily mutated, but it is remarkable to see that the mutations are only found within FR1 and FR2. It might be possible that the V_H-region of this clone and clone A5 represent chimeric products resulting from a PCR crossover event [47]. The high degree of homology of the 5' end of clones A1 and A5 with the germline sequence C57C2 would suggest a crossover with this V-region.

The effect of somatic mutation on antibody affinity could only be detected in the group of hCG/hLH cross-reactive scFv's. Clone C11, with a two- to three-fold lower affinity as clone A64, contains two mutated residues within V_H CDR3 and one in V_L CDR3. Remarkable is that this change is mediated by a relatively drastic substitution, i.e. in V_H CDR3 of the negatively charged aspartic acid (residue 100) into the polar asparagine (and with the conservative change at position 100E: alanine into proline), in V_L CDR3 of aspartic acid (residue 94) to the nonpolar glycine. Within the group of hCG specific antibodies a dramatic difference in affinity can be observed between scFv A9 and the other members of this group, but this is caused by pairing with a different light chain family.

5.3 Possible diagnostic applications of the library derived scFv's

As the major components of commercially available pregnancy tests, a large collection of murine monoclonal antibodies directed against the human chorionic gonadotropin has been built up over the last fifteen years in our laboratory at Organon Teknika. Thirty-two fusions were needed before a useful hCG-specific AB (coded 293) was obtained, due to the fact that most hybridomas produce hLH cross-reactive antibodies. We have explored phage display technology to determine if this selection-based procedure could be more efficient to select antibodies with unique hCG specificities.

From just two repertoires a complete range of scFv's with different specificities were readily selected on hCG coated onto a polystyrene surface, and with soluble biotinylated antigen. The majority of the clones selected from library G1 were directed against the β -cf and were hLH cross-reactive. As pepscan analysis revealed, these antibodies recognised peptides located in the aminoterminalus, with the common sequence RCRPI (residue 8 to 12), also present in the β -cf. Except for isoleucine and cysteine, the area is completely surface exposed (with the side chains of the arginine residues being solvent exposed) and thereby accessible for interaction with antibodies. Interestingly, this epitope contains the two arginine residues, which have been implied to confer hCG-specificity to mABs INN-hCG-2 and INN-hCG-32 [22].

Because of our interest for *in vitro* diagnostics, our attention focused on the group of hCG specific antibody fragments. The three hCG-specific scFv's coded A124 and C35, isolated from library G1 by biopanning, and A9, originated from the same library (G1), using *in solution* selection. No cross-reactivity against hLH (or hFSH) could be detected on Western blot or with surface plasmon resonance. The hFSH-CTP chimera was not detected by the antibody fragments with surface plasmon resonance, suggesting that the carboxyterminal part of the β -chain (residue number 115 to 145), unique for hCG, is not recognised. Their anti β -cf reactivity confirmed the assumption, since this degradation product misses the CTP-part. From these data we concluded that the epitope must be located within residues 6 to 40, or within the segment containing the residues 55 to 92. Therefore it may be identical to the epitope of mAB B112 [22], in which the residue number 77, asparagine, of the β -subunit confers hCG-specificity, or to the epitope mentioned before and recognised by mAB's INN-hCG-32 and INN-hCG-2 [48], for which the residues number 8 and 10 (both arginine) are involved in hCG specificity.

In respect to specificity, we believe that we have selected a number of scFv's, which might be useful for the development of a pregnancy assay. For capture of the intact heterodimer, one of the anti- α -chain specific scFv's B6 or B7 could be used, or even better the anti- α / β -heterodimer scFv B3. By increasing the avidity to $n=2$ or $n=4$ binding sites, the sensitivity might be enhanced [49, 50]. As detection antibody one of the hCG specific scFv's A9, A124 or C35 might be applied. To increase sensitivity, a dimeric format might be preferred, especially for obtaining a synergistic effect. If the hCG specific scFv's recognise the β 1 epitope from mABs INN-hCG-32 and INN-hCG-2, then it should be possible to identify a synergistic (capture) α 2 epitope

recognising antibody [48], thereby increasing the “total” affinity constant and correlated with affinity the sensitivity of the immuno-assay [51, 52].

When the affinities of the selected antibody fragments were evaluated, we found no differences with monoclonal antibodies, which are currently applied in diagnostic assays. We therefore conclude, that the anti-hCG scFv's selected from the immune repertoires might form the basis for a new generation pregnancy assay, for which the specificity, the sensitivity and the incubation times can be manipulated by application of molecular engineering technologies (reviewed in [53, 54]).

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CHAPTER 5

SELECTION OF MURINE ANTI- HIV-1 P24 ANTIBODY FRAGMENTS

Published as

Selection of recombinant, library derived antibody fragments against
p24 for Human Immunodeficiency Virus type 1 diagnostics

*Hans J.W. de Haard, Bert Kazemier, Marck J.M. Koolen,
Liekle J. Nijholt, Rob H. Melen, Bob van Gemen,
Hennie R.J.M. Hoogenboom and Jan-Willem Arends*

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1. Abstract

By application of combinatorial library technology, we generated the first recombinant antibody fragments directed against the major capsid protein p24 of Human Immunodeficiency Virus type 1 (HIV-1). A library of single-chain Fv fragments (scFvs) was constructed by using the antibody variable region genes of B-cells derived from a viral lysate-immunized mouse. Antibodies were selected by panning or by enrichment with biotinylated antigen, yielding four different families of antibody fragments. The different types of scFvs were characterised by affinity measurements, by antigen recognition on Western blots, and by pepscan analysis. The epitope of one of the scFvs is located near the residues involved in CypA binding, thereby making it an attractive candidate for therapeutic applications. Comparison of the Variable region (V-) gene sequence of a previously described monoclonal antibody reactive against this immunodominant epitope revealed the usage of the identical combination of V_H and V_K regions. Thus, this is one of the rare examples in which the original combination in a library-derived antibody fragment was retrieved. After appropriate affinity and format improvements, the best of our recombinant scFvs may form the basis for a sensitive p24 assay as a measure of viral load. In addition, anti-p24 scFvs could be expressed as intracellular antibodies (intrabodies) to aid in the treatment of HIV infections.

2. Introduction

Human Immunodeficiency Virus (HIV) infection is diagnosed by detecting virus-specific antibodies (Abs), or the virus itself, by means of p24 antigen (Ag) detection or by quantitative amplification procedures such as PCR (38) or nucleic acid sequence-based amplification (NASBA) (62), or by coculturing and subsequent virus detection procedures. During a diagnostic window of 6 to 8 weeks after infection, antibodies to HIV are undetectable, and alternative diagnostic methods would help to reduce the residual risk of transfusion transmission of HIV. Recently, the Food and Drug Administration recommended the implementation of p24 antigen tests in donor screening (20). The p24 capsid protein forms the viral core containing the single-stranded RNA genome and it is abundantly present in the virus particle. Besides the structural role of the protein in forming the core of the mature virion, the molecule is essential during viral assembly; it plays a pivotal role in viral penetration or uncoating or both, a function which may be mediated by binding of p24 to the human cellular proline rotamase cyclophilin A (4, 41, 60). With current enzyme-linked immunosorbent assays (ELISAs), the presence of p24 Ag may be assessed 5 to 14 days earlier than could an Ab response measured by anti-HIV type 1 (anti-HIV-1) or anti-HIV-2 enzyme immunoassays (8, 9, 66). In addition, the capsid protein may be considered a marker for virus replication (3, 26, 65), and its detection in an extremely sensitive

immunoassay would offer a cheap and generally applicable alternative to PCR-based assays for the diagnosis of reactivation during treatment of HIV-1 infected patients with (combinations of) nucleoside reverse transcriptase inhibitors or protease inhibitors (59, 19, 64). When reactivation, as the result of the evolution of resistant HIV mutants, is detected, treatment may be changed to other drugs. Rapid and sensitive assays that can carefully detect the presence of p24 in serum are therefore crucial for early detection and monitoring of viral replication (66).

The sensitivity and specificity of the presently used anti-p24 immunoassays are limited by the affinity of the monoclonal Abs (MAbs) used for capturing and/or detection of the Ag, although by signal amplification in combination with heat denaturation, the sensitivity can be increased to the level obtained by PCR (6). The availability of the Ab genes in recombinant anti-p24 Abs allows the improvement of affinity by mutagenesis methods, as well as the engineering of avidity, thereby helping to improve the sensitivity of early virus detection. During *in vivo* maturation, the obtained affinities are limited by the off-rate, i.e., the rate at which the Ab-Ag complex dissociates. The off-rate of *in vivo*-matured Abs is in the order of 10^{-3} to 10^{-4} s⁻¹, which permits endocytosis of membrane bound antibody-antigen complexes on B-cells (21). However, *in vitro* maturation with phage display allows the selection of Abs with lower off-rates, leading to affinities in the picomolar range (1, 57). Besides the obvious diagnostic application, it may also be possible to use anti-p24 single-chain Fv fragments (scFvs) for therapy. By expression with a retention signal for the endoplasmatic reticulum, the scFvs may interfere with virus assembly in the infected cells, as was demonstrated with anti-gp120 (12) and anti-Tat (45) Abs. For intracellular expression, even murine Abs might be applied in humans: a human anti-mouse antibody (HAMA) response can not be induced, since the fragments are shielded from the immune system.

In this context, we aimed to isolate anti-p24 scFvs from a phage Ab library made from a mouse immunized with HIV-1 viral lysate. Affinities were determined with surface plasmon resonance and compared with those of MAbs currently used in p24 assays. The specificity of the selected Ab fragments was determined by ELISA, Western blotting, and pepscan analysis. One of the recombinant Ab fragments recognized an epitope, which was also detected by a previously described and sequenced murine Mab. Both the recombinant Ab and the hybridoma-derived Ab have nearly identical heavy and light chain variable regions, and, as such, the selected scFv D2 is one of the first examples in which the original pairing of both V_H and V_L was maintained, in spite of the very low probability of finding this in a combinatorial library (23).

These newly described anti-p24 recombinant scFvs offer a starting point for Ab affinity maturation and engineering (for review see (30, 67, 68)), and may be useful as intracellular Abs (intrabodies) for HIV therapy.

3. Materials and methods

3.1 Library construction

A Balb/c mouse was immunized with 100 μ g of protein from a viral lysate, which had been prepared from infected H9 cells (52) and purified by ultracentrifugation, plus Freund's complete adjuvant. After 6 weeks, a booster with 100 μ g lysate proteins plus incomplete adjuvant was administered. Three days later, the animal was sacrificed and the spleen was removed. Upon extraction of the spleen, 20 μ g of total RNA was isolated (13) and 5 μ g of this was transcribed into random-primed cDNA.

The immunoglobulin G1 (IgG1)-derived V_H - and V_K -encoding DNA fragments as well as the linker fragment (the latter was cloned in pUC19 by using synthetic oligos, which encoded the 15-residue linker described by Huston and colleagues (33)) were obtained by amplification (Amplitaq; Perkin-Elmer Cetus) with the primers (14) listed in Table 1. The fragments were purified from an agarose gel with the QIAEX kit (Qiagen, Hilden, Germany), and assembled in splicing by overlap extension-PCR (32).

The assembled scFv-encoding DNA fragments were also purified from the gel, digested with *Sfi*I and *Not*I (Pharmacia, Lund, Sweden), and cloned into the phagemid vector pV1, which is identical to pHEN1 (31) but with a FLAG-tag (53) instead of a Myc-tag. Transformations were performed by electroporation in JM101 (17). The library consisted of 10^7 clones, of which 60 to 70% contained a complete insert as was deduced from PCR analysis.

3.2 Selection of libraries

The rescue of phagemid particles was performed as was described before (43). The capsid protein p24, expressed as full-length product in *Escherichia coli* under control of the inducible *lac* promoter (constructed at Organon Teknika [3a]) and encoding the HIV-1 strain RF-sequence, was affinity purified with MAb 39B (generated at Organon Teknika [unpublished data]). A six-well tissue culture plate (Costar, Cambridge MA., USA) was coated with Ag at a concentration of 3 μ g/ml in 50 mM NaHCO_3 pH9.6. Selection, washing, and elution were performed as described elsewhere (43).

For the construction of a light chain shuffling library of clone D2, the V_H segment was amplified on plasmid DNA. The V_H fragment was recombined with the linkers and the corresponding kappa light chain fragments, initially used for the generation of the library, yielding the shuffling library containing 10^6 clones. Selection was performed with p24 biotinylated with *N*-hydroxy-succinimide-biotin (Pierce, Rockford, Ill.); phage Ag complexes were captured with streptavidin-coated paramagnetic beads (Promega, Madison, Wis.).

Table 1. Oligonucleotide primers used for construction of the library and sequencing

purpose	primer name	sequence
VH	VH1back (a)	5' aatag <u>gccagccggcc</u> atggcc(C/G)AGGTTCA(A/G)(G/C)TGCAGCAG(C/T)CTGG3'
	VH2back (a)	5' aatag <u>gccagccggcc</u> atggccGAGGTG(C/A)AGCTT(G/C)T(G/C)GAGTCTGG3'
	VH3back (a)	5' aatag <u>gccagccggcc</u> atggccCCGA(G/T)GTGCAGCTTCAGGAGTCAGG3'
	VHfor	5' CCTGAGGAGACGGTGACC(A/G/C)(G/T)GG(G/T)(C/T)CC3'
	IgG1-CHfor	5'TGGATAGACAGATGGGGG3'
VK	VK1back	5' ggatctGACATC(C/A)AGATGACTCAG(T/A)CT3'
	VK2back	5' ggatctGATATTGTGATGACGCAG(G/A)(C/A)T3'
	VK3back	5' ggatct(G/A)ACATTGTG(C/A)TGTCACAGTCTCC3'
	VK4back	5' ggatctGAAA(A/T)T(G/T)TTCTACCCAGTCTCC3'
	VK5back	5' ggatctGATATT(G/T)TGATGACGCAG(C/T)CTGC3'
	VKfor (a)	5' aatggaattccggcgccgcCGGTTTTA(T/G)(T/C)TCCAGCTT(G/T)GT(G/C)CC3'
	CKfor	5' AGTTGGTGCAGCATCAGCC3'
linker	VHLINKback	5'GGTCACCGTCTCCTCAgg3'
	VK1LINKfor	5'AG(A/T)CTGAGTCATCT(G/T)GATGTCagatcc3'
	VK2LINKfor	5'CTGCGTCATCAAAATATCagatcc3'
	VK3LINKfor	5'GGAGACTGTGACA(G/T)CACAATGT(C/T)agatcc3'
	VK4LINKfor	5'GGAGACTGGGTGAGAA(C/A)A(T/A)TTTCAGATagatcc3'
	VK5LINKfor	5'GCAG(G/A)CTGCGTCATCA(C/A)AATATCagatcc3'
sequencing	geneIII	5'TGAATTTTCTGTATGAGG3'
	linkfwd	5'CTCGGGCGGTGGTGGGTC3'
	linkrev	5'GACCCACCAACGCCCGAG3'

^a The *Sfi*I sites in the VHback primer sequences and the *Nci*I site in the VKfor primer sequence are underlined. Nucleotides represented as uppercase characters are carried by the V gene; those in lowercase characters are carried by the vector.

3.3 Screening and sequencing of clones

Soluble scFv was produced from individual clones by growth in V-shaped microtiter plates in 2*TYE medium as described before (43). The described method was applied for constructs transformed in the nonsuppressor strain TOP F', as well as in the suppressor strain JM101. Suppression is incomplete and results in leakage of free scFv in the culture medium, while the expression of geneIII makes the cellular membranes more permeable (5), leading to higher concentrations of Ab fragments in the medium. Microtiter plates were coated with 3 µg of recombinant p24 per ml (see above) or 5 µg of viral lysate per ml in 50 mM NaHCO₃ (pH 9.6) for 16 h at room temperature. The plates were blocked for 2 h at room temperature with bovine serum albumine (BSA; 0.2 % [wt/vol] in 0.1 M Tris [pH 7.4]-30 mM KI). The culture

supernatant was diluted fivefold in sample diluent (phosphate-buffered saline [PBS], 20 % normal goat serum; 1.1 % Triton X-100). After incubation at room temperature for 2 h, the plates were washed four times with PBS-Tween 20 (0.05%), and bound scFv was detected with a mixture of a 1/4,000 dilution of anti-FLAG Ab M2 (Kodak IBI, New Haven, Conn.), and a 1/2,000 dilution of anti-mouse Ab-horseradish peroxidase conjugate (DAKO, Glostrup, Denmark). Following a 1-h incubation and washing as before, staining was performed with tetramethylbenzidine and ureaperoxide as the substrate and stopped by adding an equal volume of 1M H₂SO₄; the optical density was measured at 450 nm.

Clones giving positive signals and with a unique *Bst*NI fingerprint (New England Biolabs, Beverly, Mass.) were analyzed by sequencing with the T7 sequencing kit (Pharmacia) with the M13rev primer, the geneIII primer, and two primers located in the linker (LINKfwd and LINKrev) (Table 1). Plasmid DNA was purified with the QIAGEN kit (Qiagen).

3.4 Characterization of scFvs

For the preparation of periplasmic fractions by the borate-buffered saline shock procedure (58), clones obtained by transfection to the nonsuppressor strain TOP F' were cultured on 50-ml scale and induced as described previously (43). The fractions were analyzed on 15% polyacrylamide gels and then blotted onto nitrocellulose. After blocking with a skim milk solution (5% [wt/vol] in Tris-buffered saline [TBS]), the scFv was detected with anti-FLAG M2 diluted 1/4,000 in TBS-0.05% Tween (TBST). After a 2-h incubation period, the anti-FLAG was detected with anti-mouse Ab-horseradish peroxidase and the ECL detection kit (Amersham, Buckinghamshire, United Kingdom).

For the purification of scFv, refolding was used in combination with affinity chromatography on p24 columns. The pelleted cells from a 50-ml culture were resuspended in 8 ml of an 8 M urea solution (in PBS) and sonicated. The mixture was rotated head-over-head for 30 min, and insoluble material was removed (centrifugation for 30 min at 13,000xg). The supernatant was dialyzed against PBS with four buffer changes. Insoluble proteins were removed by centrifugation, and after passing through a 0.2- μ m-pore-size filter, the flowthrough was immediately loaded on a p24 column (bed volume, 0.3 ml). The column material was prepared by coupling 8.4 mg of protein to 1 g Tresyl sepharose in accordance with the supplier's instructions (Pierce). Washing and elution were performed as in the panning procedure. The yield was determined by measuring the optical density at 280 nm, assuming that an scFv has a molar extinction coefficient ($E^{1\%}_{280\text{nm}}$) of 14.3 (27).

In order to establish the valencies of the scFvs, the molecular weight of the antigen binding molecules were determined by gel filtration (27) on a Superdex 75HR column (Pharmacia), which was calibrated with a mixture of BSA, chymotrypsin, ovalbumin, and RNase A (Pharmacia). As a control, an anti-human chorionic gonadotropin (anti-hCG) scFv selected from a murine immune library was used (unpublished data). Either 20% of a periplasmic fraction from

a 50-ml culture or affinity-purified scFv was injected onto a calibrated column. Fractions (0.5 ml) were collected and analyzed on Western blots.

Epitope mapping was performed with the pepscan method (22). Overlapping dodecapeptides of p24 from HIV strain ANT70, coupled to the solid support and obtained from the Department of Molecular Recognition of the Institute for Animal Science and Health (IDO-DLO), were tested with the scFv. The periplasmic fraction of clone D2 was diluted 100-fold in super Q buffer (PBS containing 5% [vol/vol] horse serum, 5% [wt/vol] BSA and 1% [vol/vol] Tween 20) to a concentration of 80 ng/ml. After 16 h of incubation at 4°C, scFv bound to peptides on the solid support was detected as described above.

3.5 Affinity measurements in solution

The affinities measured by the "in solution" method were determined with surface plasmon resonance on BIAcore (Biacore AB, Uppsala, Sweden) in accordance with the suggested procedures described in the BIAevaluation software. A high-density p24-coated (3777 Response Units [RUs] of immobilized Ag) CM-chip (Biacore AB) was prepared by covalent coupling according to the supplier's recommendations. A fixed amount of scFv or MAb, yielding a signal of between 1,000 and 1,500 RUs when injected onto the p24 chip, was mixed with a variable amount of Ag in a volume of 60 µl by using HEPES- buffered saline (10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl, 0.05% surfactant P20 [pH 7.4]) as dilution buffer. After a 1-h period, needed to achieve equilibrium, the samples were injected. For the quantification of free AB, a standard curve was prepared by injection of a dilution series of the tested Ab on the same flow cell (37).

3.6 Nucleotide sequence accession numbers

The GenBank accession numbers for the sequences of the antibodies reported in this study are as follows: D2 V_H, AF083186; A2 V_H, AF081541; A3 V_H, AF081545; A5 V_H, AF081543; D1 V_H, AF083189; D2 V_L, AF083188; A2 V_L, AF081542; A3 V_L, AF083185; A5 V_L, AF081544, D1 V_L, AF083187.

4. Results

4.1 Library construction and selections

Our major aim was to evaluate the library technology as a substitute for hybridoma technology for the generation of anti-HIV-1 Abs, which might be useful for *in vitro* diagnostics. Therefore, we immunized a mouse with a lysate prepared from HIV-1 infected H9 cells (52). After amplification of the V-genes of the murine spleen B cells and cloning in the phagemid vector pVI, a library containing 10⁷ clones was obtained. Seventy-five percent of the clones

expressed scFvs, as was determined by PCR screening and by inspecting the morphology of the colonies: clones which express gene III (fusions) are usually visible as flat colonies, while those having an interrupted frame exhibited a more solid appearance (14a).

The library was subjected to four rounds of panning on recombinant p24. Four rounds of panning produced a 100-fold enrichment in the number of eluted phages, indicating the presence of Ag-specific phage Abs. The scFvs produced by 48 individual clones after round 4 were analyzed by ELISA (43). Seventy percent of the tested scFvs produced high signals on viral lysate and p24 Ag, whereas no responses were found on BSA. After 24 clones were DNA fingerprinted by *Bst*NI digestion (43), two different patterns were identified; five clones representing the two patterns were selected for sequence analysis and further binding analysis. All five clones analyzed had minor differences in sequences and were classified as scFv D2 (Fig. 1).

Immune libraries usually contain a very diverse set of Ag-specific Abs (14, 44), thus, we modified our method of selection to retrieve phage Abs other than the ones found before. Selections were repeated with soluble (biotinylated) recombinant p24 (Sol-p24) and with p24-coated magnetic particles (Bead-p24). After four rounds, enrichment factors of 100 and 10,000 were found for Sol-p24 and Bead-p24, respectively. Concomitant with this enrichment in the absolute number of eluted phages, we found an enrichment in the fraction of clones producing scFv, as determined by colony morphology. The percentage of scFv-producing clones increased from 50 to 75% of the clones before selection and after round 1 to more than 99% after round 4. At this stage, 8 of 11 clones of Sol-p24 and 12 of 12 of Bead-p24 contained a complete insert, whereas *Bst*NI fingerprinting revealed four different but closely related clones for Sol-p24 and only one type for Bead-p24. The four Sol-p24 scFv clones reacted positively on p24 and negatively on BSA, while the Bead-p24 clones gave high-level responses on p24 and BSA. Obviously, panning with the beads resulted in the isolation of an scFv specific for the blocking reagent BSA, which was also used for blocking of the microtiter plates, which explains the positive signals in the p24 ELISA. The selection of BSA-specific clones can be explained by the presence of this Ag in the viral lysate used for immunization and its use for blocking the beads and the microtiter wells. We chose three different p24 binders of Sol-p24 and the Bead-p24 BSA binder for sequencing and further characterization. For Sol-p24, the clones designated A2, A3 and A5 were used; for Bead-p24, the clone was named D1.

Figure 1 (on next page). Deduced amino acid sequences of the variable heavy and light chain regions and germline origin of the p24 Abs derived from the murine library. Identical residues are indicated by dashes, while different residues are shown with uppercase characters and primer-encoded differences are indicated by lowercase characters.

A. Heavy chains

Antigen	Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
		QVQLQQSGPDLVKPGASVKISCKASGYSFT	GYMH	WVKQSHGKSLEWIG	RVNPNNGGTSYNQKFKG	KAILTVDKSSNTAYMELRSLTSEDSAVYYCAT	AT	FGY WQGGTPVTVSS
	#D2	e-----	D-L--	--N--P--	-----A-----	-----I-----	---	-----I-----a
p24	#BB128	e-----	D-L--	--N--P--	-----A-----	-----I-----	---	-----I-----a
	#A2	-----E-----L--L--	--T-N--	--N--	LI--Y--D-RN--	--T--S--	--R	DPYYGSSWY-DV --A-----
	C57C2	e-----E-----	--N--	--PE--	EI-----	--T--S--	---	---
	#A3	EVQLQESGPDLVKPGASVKISCKLSGYTFT	DYNH	WVKQSHGKSLEWIG	YIYPSNGDTGYNQKFKT	KATLTVDNSSSTAYMELRSPTSEDSAVYYCAR	DPNYFGSSHSFAY	WGECTLTVTVSS
	#A5	-----G-----T--	---	NG--	-----G-----	-----L-----	---	-----Q--m-----
p24	C57/C27	-----q-----A--	--YMD--	---	D-N-N--G-I--	-----K-----	---	---
	#D1	QVQLQQSGPDLVKPGASVKISCKTSGYTFT	EYTMH	WVKQSHGKSLEWIG	GINPYNGDTTYNQKFKG	KATLTVDKSSSTAYMELRSLTSEDSAVPYCAR	RGYGYRYTT	MDY WGGKTLTVTVSS
BSA	C57C2	e-----A-----	D-Y-N	---	D--N--G-S--I--	-----	---	---

B. Light chains

Antigen	Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
		DIQMTQSPASLSVSVGETVTITC	RASENIY	SNLA WYQQKQKSPQLLVY	NAKTSAE	GVPSRFSGSGSGTQFSLKINSQPQEDFGSYIC	QHMYGTPFT	FGSGTKLELKR
	#A3	-----k-----t-----	---	---	-----L-----	-----L-----	---	-----A-----
	#A5	-----t-----A-----	---	-Y--	-----L-----	-----L-----	-----I-P--	---
p24	#A2	-----A-----	---	NY--	-----L-D--	-----Y-----	--FWS--	---
	k2/MMIG27	-----A-----	--G-H	---	---	---	---	---
	#D2	DIVMTQTPLTSLSVTIGQPASISC	KSSQSLDSDGKTYLN	WLLQRPGQSPKRLIY	LVSCLDS	GVPRFTSGSGSDTFTLKISRVEAEDLGVYYC	WQGHFFWT	FGGGTKLEIKR
	#BB128	-V-----	---	-----Q--	---	---	-----Y-----	---
p24	70/3	-V-----	---	---	---	---	---	---
	#D1	DIVMSQSPASLAVSLGQKATISC	RASEVD	SYGNSFMH WYQQKPGQPPKLLIY	LASNLES	GVPARFSGSGSRDFTLTIDPVEADDAATYYC	QQNNEDPWT	FGGGTKLELKR
BSA	21B	n-lt-----R-----	---	---	---	---	---	---

4.2 Characterization of isolated scFvs

Figure 1 shows the deduced amino acid sequences of the V_H and V_L gene products of the clones derived from panning the murine library with Ag coated polystyrene plates (D2) and with biotinylated Ag (A2, A3 and A5) together with the most related mouse germline analogues (V regions). The sequence of the BSA binding antibody (D1) selected with the beads is also included.

Figure 2. Western blot detection of p24 by scFvs and mouse polyclonal Abs.

(A) Detection with scFv D2 (left panel) and A3 (right panel). Viral lysate (lanes 1), BSA as a negative control (lanes 2), and purified recombinant p24 (lanes 3) were analyzed on a 12% gel. The band indicated with an asterisk represents the dimer of p24, present in the recombinant and in the viral product.

(B) Blot analysis of viral lysate with polyclonal serum (strips labeled with +) obtained from the mouse used for library construction (left blot made with an 8% gel and the right one with a 12% gel). Amido black-stained markers (lanes labeled M) are shown. The Gag precursor p55 and its derivative p24, the envelope protein gp120, and the polymerase p31 are indicated with arrows. The slow-migrating protein indicated with the top-unlabeled arrow in the left blot is probably the envelope precursor gp160. The other protein marked with the bottom-unlabeled arrow, is a cleavage product of Gag, which is also recognized by scFv D2 (Fig. 2A).

The four anti-p24 scFvs use highly homologous V_H gene segments. They belong to class IIA according to Kabat's classification (36) and use two germline gene segments only. Clones A2 and D2 and clones A3 and A5 can be grouped in two classes depending on the germline used. D2 has a divergent and shorter CDR3 than clone A2 and thus is clonally not related. The

light chain of clone D2 could be matched adequately with a germline segment, and is very different from the A2 light chain. The kappa light chains of the three Abs A2, A3 and A5 use a nearly identical V_{κ} gene segment, derived from the germline segment coded k2/MMIG27 (for classification and the codes used in Fig. 1, see "Germline gene directories of the mouse" homepage (http://www.ibt.unam.mx/~almagro/V_mice.html)).

The specificities of the scFvs were determined by Western blot analysis (Fig. 2). Periplasmic fractions containing scFv were used for the detection of viral and recombinant p24. Two different recognition patterns were obtained. Abs A2, A3, and A5 reacted with (the reduced form of) p24, in a viral lysate, and as a recombinant product (for example, the blot incubated with scFv A3 is shown in Fig. 2A). ScFv D2 also recognizes p24, but it also detects the viral Gag polyprotein p55 (Fig. 2A, left panel, lane 1) from which the matrix protein p17, the nucleocapsid p7, and the capsid protein p24 are proteolytically processed. Another product with a molecular weight of approximately 40 kDa is visible. This protein seems also to be recognized by polyclonal serum (Fig. 2B) obtained from the mouse that was used for the construction of the library. The p24 dimer, which is the predominant form of the native protein as was observed by gel filtration (results not shown), might also be detected with mouse serum (Fig. 2B, left panel, band between p55 and the 40-kDa derivative). Indeed, the native p24 protein can form a complex of oligomers in solution, including dimers, tetramers, dodecamers, spheres, fibers and tubes (18). An intense reactivity of the original murine polyclonal serum against p24 and its precursors can be observed, while the mouse seems to have a lower Ab titer against the envelope protein gp120. Thus, the original library was biased in its immune response towards p24, which is probably caused by the abundance of the antigen in the viral lysate used for immunization (10, 63).

4.3 D2 is a Diabody

After performing the sequence analysis of clone D2, selected on immobilized p24, we noticed that it contained a linker consisting of 2 amino acids (GlySer) rather than 15 amino acids ((Gly₄Ser)₃). As suggested in the literature (29), shortened linkers may lead to the formation of dimeric molecules with two functional binding sites, the so-called 'diabodies'. The shorter linker in clone D2 might therefore be responsible for the formation of a bivalent scFv molecule, and this type of molecule might be strongly enriched over the other anti-p24 scFvs by its greater avidity advantage in the panning selection. We generated a number of D2 derivatives and characterized their behavior.

From a light chain shuffling library, we selected a derivative of clone D2, that had an identical light chain and a complete 15-amino-acid linker; the clone was designated D2/15. We examined the molecular weight of the antibody fragments present in the periplasmic space of the producing bacteria by gel filtration on a calibrated Superdex 75HR column (27): the obtained

chromatogram of clone D2 is shown in Fig. 3A. The scFv with a two-amino-acid linker emerged from the column as a single peak with a molecular weight of 45 kDa, as could be deduced from Western blot analysis with the fractions collected during gel filtration (Fig. 3B). Both scFvs, D2/15 and a control anti-hCG Ab fragment with a 15-residue linker, gave two peaks: the dimeric fraction at 45 kDa and a monomeric fraction at 25 kDa.

By chain shuffling the light chain of the D2 diabody, clone D2/15 was isolated by using an excess of Ag during selection. By increasing the stringency of selection, i.e. decreasing the Ag concentration from 100 nM to 100 pM, the monomeric clone D2/15 was completely lost and the selected population was overtaken by (avid) diabodies. We retrieved no D2 derivatives with other light chains, not even the one of clone A2, in spite of its highly related V_H gene, as was also reported by others (56). However, it should be emphasized that the diabody's CDR3 sequence is completely different from clone A2 in length as well as in sequence. Due to the close proximity of H3 to the light chain variable-region loops, this may determine the pairing light

Figure 3. Gel filtration of periplasmic fractions on Superdex 75HR and analysis of the collected fractions on Western blot with anti-FLAG M2.

(A) Chromatogram, obtained by plotting A_{280} against the fraction number, illustrating the separation of the periplasmic proteins from clone D2. The retention times of the marker proteins are indicated with arrows.

(B) Western blot analysis (15% gel) of the collected fractions (fraction number is shown above the blots) obtained from gel filtration of anti-p24 clones and of an anti-hCG scFv-producing clone. Two peak fractions can be discriminated in the scFvs having a complete linker (the derivative D2/15 and the anti-hCG): a main peak of the monomer (fraction 20; molecular mass 25 kDa) and a dimer peak (fraction 17; molecular mass 45 kDa). ScFv of clone D2 emerged from the column in a single peak corresponding to the dimeric product.

chain. In the course of the shuffling experiment, we isolated one clone, D2/L3mut, which appeared to have a 15-residue linker but still produced predominantly dimeric scFvs. Sequencing revealed a single mutation from the D2 sequence, of residue W (shown in boldface type in Fig. 1) to Y, in the third hypervariable region of the light chain. Since this region of the Ab is in close contact with the V_H region, the mutation may destabilize the V_H - V_L association, thereby promoting dissociation and intermolecular pairing (56).

4.4 Affinity measurements

For affinity measurement, the scFvs were purified by affinity chromatography on Ag columns. The scFv fragments were refolded after denaturation of all cell proteins with urea by subsequent dialysis against PBS. Although the efficiency of refolding is limited, the yield of functional scFv is much higher than in periplasmic fractions obtained by the osmotic shock procedure (58). The purity was analyzed on a Coomassie blue-stained gel (data not shown).

All purified scFvs were analyzed by gel filtration chromatography, as was already performed with the periplasmic fraction of clone D2 (Fig. 3); the purified product of the D2 diabody contains dimers only, while the analogue with the complete linker, D2/15, is

Table 2. Affinities of the anti-p24 MAbs and scFvs

Ab type	Ab	form ^a	linker length (aa) ^b	K_d (M)
MAb	5B4			3.6×10^{-8}
	34A			8.0×10^{-9}
	39B			8.0×10^{-9}
scFv	A2	nd ^c	15	2.0×10^{-6}
	A3	monomer	15	2.5×10^{-8}
	A5	monomer	15	3.0×10^{-8}
	D2	dimer	2	1.0×10^{-6}
	D2/15	monomer	15	3.0×10^{-6}
	D2L3mut ^d	dimer	15	1.3×10^{-6}

^a As determined by gel filtration.

^b aa, amino acids.

^c ND, not determined.

^d This clone is identical to clone D2 but with a complete linker and a single mutation within CDR3 of V_K (see text).

predominantly monomeric. Clones A3 and A5 produce monomeric scFv fragments only (Table 2).

We compared the affinities of the library-derived scFvs with the binding strength of hybridoma-produced MABs. We chose to measure the kinetics of binding by surface plasmon resonance on a BIAcore machine. When using Ag immobilized on sensorchips, we always observed biphasic dissociations, probably caused by the oligomerization of p24 (18), resulting in a heterogeneous coat. The abnormal sensorgrams were also noted before by others (25). Therefore, the affinity was determined in solution (47), by injecting a preestablished equilibrium mixture of a fixed amount of Ab and a variable amount of Ag on a sensorchip with a high-density coat of Ag. Under mass transfer limiting conditions, the amount of free Ab (Ab_{free}) is proportional to the binding rate. Subsequently the K_d is obtained by plotting the concentration of Ab_{free} (or the fraction Ab_{free}/Ab_0 ; Ab_0 is the concentration Ab when no Ag has been added) against the total concentration of Ag. In Fig. 4, the binding curves derived from the MABs and scFvs are shown; Table 2 gives the corresponding K_d s. The affinities of the MABs 34A and 39B are threefold higher than those of scFvs A3 and A5, both selected on biotinylated Ag, which in turn have slightly higher affinities than MAB 5B4. The D2 diabody, selected on immobilized p24 via panning, has a much lower affinity (micromolar range). The monomeric version of D2, D2/15, has a threefold-lower affinity than that of its dimeric counterpart, demonstrating that the effect of avidity on the apparent affinity determined in this assay is limited. Finally, clone D2/L3mut, which expressed mainly dimeric scFv (as gel filtration of affinity-purified scFv revealed [results not shown]) had an affinity approaching that of clone D2.

Figure 4. The percentage of Ab_{free} as a function of Ag concentration (logarithmic scale). The amount of Ab_{free} (i.e., not bound to soluble Ag) was determined by surface plasmon resonance and related to the input amount Ab (no Ag added). The K_d was derived from the plots at 50% binding.

4.5 Epitope recognition

Pepscan analysis (22) was used in the identification of the epitopes of the scFvs. Although all recombinant fragments recognized denatured Ag on Western blot, only the diabody type D2 gave a good response. The results obtained with the overlapping dodecapeptides based on the

Figure 5. Pepscan results obtained with dodecapeptides of p24 of HIV-1 strain ANT70 with scFv D2 (A) and serum Abs of an HIV-1 patient (B).

The numbering on the horizontal axis corresponds with the amino terminal amino acid of the peptide; the first peptide starts with a Q at -3 relative to the cleavage site of p17 and p24, the last peptide (no. 240) ends with A located at -3 relative to the cleavage site of p2 with p7. On the vertical axis, the optical density at 450 nm obtained for each peptide in ELISA has been plotted. The common sequence recognized by the human serum and scFv D2, starting with peptide 76 and ending with peptide 82, is indicated in panel B by solid bars.

sequence of HIV-1 subtype O strain ANT70 and the scFv-containing periplasmic fraction are shown in Fig. 5A. The Ab reacted with the peptides starting with the sequence INEEAVEEWDRTN and ending with EWDRTNPPPVGP, thereby having in common the sequence EWDRTN. This sequence is also recognised by serum Abs from an HIV-1 patient (Fig. 5B), indicating that the immune system of the mouse recognizes the same epitope on the immunized Ag as that of humans after virus infection. It should be noted that the p24 used for immunization and selection is derived from strain IIIB, while the pepscan is based on strain ANT70. For the A2, A3, and A5 Abs, the pepscan based on the ANT70 and the IIIB sequences did not reveal any unique epitopes (results not shown), indicating that these Abs may recognize a conformation-sensitive epitope.

Inspection of the HIV-1 B-cell epitope database (Los Alamos) showed us that several MAbs that share the epitope with scFv #D2 have been found. From one of these, hybridoma BB128y (61), the V gene sequence contains a nearly identical heavy chain variable region, with a remarkably short H3 loop, and, even more surprisingly, a light chain variable region with only one residue difference in the germline-encoded region from D2 (Fig. 1).

5. Discussion

5.1 Isolation of different sets of anti-p24 Abs dependent on the selection procedure

When we used the combinatorial library technology for the generation of MAbs, we found that the selection method determines to a great extent what type of Ab will be found. By using Ag-coated polystyrene, the high Ag density probably favors the selection of scFvs which are avid, exemplified by the selection of the D2 clone, while selection with soluble Ag leads to the isolation of monomeric Ab fragments produced by clones A2, A3, and A5. In the course of the assembly reaction between V_H , linker, and V_L to generate scFvs with 15-residue linkers (14), mispriming may infrequently lead to short-linker versions of V_H and V_L as reported previously (42). In the resulting Abs with a 5- or 10-residue linker, structural constraints prevent the intramolecular pairing of V_H and V_L . Instead, the product forms an intermolecular pair with another V_H - V_L fusion product, yielding a bivalent scFv molecule, the so-called diabody (29, 44). The D2 diabody is heavily dependent on avidity for its selection; when a selection procedure with soluble p24 is used, the avidity effect is less pronounced and other clones (A3 and A5) are selected on the basis of affinity. The avidity effect may be more pronounced also by the intrinsic aggregation behavior of p24, which upon coating may lead to epitopes that are spatially ordered such that they are more prone to cross-linking by scFv Abs. Alternatively, the epitope recognized by clones A3 and A5 might be shielded or modified on p24 adsorbed onto polystyrene, although this hypothesis was not supported by the high ELISA signals. Our data indicate that selections should be carefully fine-tuned to retrieve the desired Abs. To obtain a wider panel of anti-p24

Abs, the various selection procedures used here may be altered or extended, for example, by using epitope-shielding Abs as described elsewhere (16).

The affinities of the Abs were determined with an in solution method. The multimerization behavior of p24 complicated the affinity determination by direct kinetic measurement, while the alternative assay gave reliable data with only a minor effect of avidity. The affinities of the monomeric scFvs A3 and A5 found by selection with soluble Ag are in the nanomolar range and are slightly better than the affinity of one of the studied MAb, which was taken from a panel of hybridoma-produced MAb that are currently used in diagnostic assays. Their affinity is still threefold lower than of the best MAb tested, but this might be due to the avidity of the MAb. Indeed, a threefold difference was also found between the dimeric and the monomeric versions of clone D2.

The present study showed that Ab fragments obtained from the murine library have comparable affinities to those of their hybridoma-derived analogues. For obtaining high-affinity Abs useful as diagnostic reagents, avidity may be recruited, for example, by making diabodies (29), scFv trimers (39), or tetramers (for a review, see reference 51). The affinity of the best selected antibodies, A3 and A5, may be improved, for example, by targeted mutagenesis (1, 30, 57). Finally, additional alternative selections should be employed to further explore the diversity of the murine phage library that was constructed.

5.2 D2 recognizes an immunodominant epitope closely located at the CypA binding site

All selected scFvs recognized linear epitopes of p24, as was concluded from Western blot studies. p24 expressed in *E. coli* as well as Ag, derived from cultured virus and presented in a reduced form was reactive on Western blot. Pepscan analysis with overlapping dodecapeptides yielded the epitope of the scFv produced by clone D2. The three-dimensional structure of the amino-terminal core domain of the HIV-1 capsid protein is known (24); it reveals the presence of an arrowhead-shaped domain, with seven helices and two β hairpins, and an exposed partially ordered loop involved in binding cyclophilin A. Recently, the binding domain was analyzed in more detail by crystallization of a peptide fragment of p24 with cyclophilin A (69), indicating the direct interaction of residues Ala 88, Gly 89, Pro 90, and Ile 91 with residues from the cellular enzyme. The epitope of D2 is located within the carboxy-terminal part of helix IV, ending at His 84 (or Leu in strain IIB), and it is separated by three residues from the residues interacting with cyclophilin A.

The interaction of p24 with cyclophilin A appears to result in the packaging of approximately 200 copies of CypA into each HIV-1 virion. Although the function of CypA remains unclear, virions lacking the enzyme are known to be poorly infectious (7). Cyclosporins capable of binding to cyclophilin A are effective in inhibiting virus replication, as was reported before (4, 41, 60). Agents that interfere with the CypA binding function of p24 may be useful in reducing viral load; application of our scFv D2 as intracellularly expressed Ab may therefore

prevent binding of the enzyme in infected cells, rendering the progeny virus less infectious or noninfectious, as was done successfully with anti-Tat (45) and anti-gp120 (12) Abs.

5.3 Retained V_H - V_L pairing

Several epitopes have been mapped within the CypA loop region, which by its exposed orientation is accessible for reaction with Abs. Other MABs that recognize the same epitope have been reported (28, 34, 48, 49, 50, 54, 55). In addition, the structure of a complex between an anti-p24 Ab and its Ag was determined with an Ab with nearly identical epitope recognition as Ab D2 (46). Moreover, the epitope is also reactive with human Abs, as indicated by our pepscan with serum from an HIV-1-infected patient and as was also concluded in a study in which the peptide was incorporated in a diagnostic assay (35).

Despite the recognition of the same epitope by the Abs mentioned above, their heavy chain V genes have little homology to each other, except that they both use a CDR3 of the same size and a nearly identical CDR1. The most striking resemblance exists between scFv D2 and anti-p24 MAB BB128 γ (61), which have almost the identical V_H and V_K sequence (differences of six and two amino acid residues, respectively). Clones isolated from a V_K light chain shuffling library were found to have the original MAB light chain sequence, including the tyrosine (replacing the tryptophan present in clone D2), indicating a favorable selection for original pairings. It has been argued that the chances of retrieving the original pairing from a combinatorial library are very low, as the result of RNA isolation (67), and that it is particularly difficult to retrieve the original light chain (2). During the early days of combinatorial library construction with the bacteriophage lambda system, an original V_H - V_L combination identical to the one found in a hybridoma Ab was reported (11). However, the identity was less pronounced than that with our p24 scFv: in the partially sequenced V_H , containing CDR2, FR3, and a part of FR2, there were eight mismatches present and the light chain comprised four differences. Moreover, the V_H could pair with a different V_L , as was also found by Barbas and colleagues (2). Considering the results from the light chain shuffling library, the D2 V_H is strictly monogamous, i.e., it can pair with its original light chain only. When a mouse has 10^6 different B cells, a library with at least 10^{12} clones must be made to have a chance to identify an original V_H - V_L combination. After immunisation, the number of anti-p24-expressing B lymphocytes will be larger, but still a rather huge library would be needed for finding original combinations. One of the reasons that we isolated the original clone may be because of a high titer of these Abs, since pepscan analysis with the polyclonal mouse serum gave a major response on the peptides containing the EWDRTH sequence (data not shown).

5.3 Diagnostic applications

The selected anti-p24 Abs could be used in a sensitive p24 Ag assay in which HIV-1 infection could be detected in an early phase (20). The correlation between affinity and

sensitivity in ELISA has been clearly demonstrated (15, 40). The affinities of the selected scFvs approach the values of MAbs, which can detect 5 to 10 pg p24 per ml of serum, when combined in a capture assay. An exception is scFv D2, with its micromolar level of affinity. It has been reported that the closely related MAb BB128γ is a synergistic Ab, meaning that its affinity is enhanced dramatically (up to 1.000-fold) when combined with MAbs, which recognize epitopes on the opposite side of the Ag. In vitro affinity maturation (1, 30, 57) of the Abs might improve the sensitivity to a level comparable with that of PCR detection (6). The format of the Abs could be adapted to their application; for instance, a high-affinity monovalent fragment might be useful for capturing Ag, while a multivalent scFv might be more suitable for the sensitive detection of high-density Ag arrested on a solid surface. Therefore, the application of engineered Ab fragments should be very useful for the development of new generations of diagnostics.

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CHAPTER 6

SELECTION OF HUMAN ANTI- HIV-1 ENVELOPE SINGLE CHAIN ANTIBODIES

Published as

Selection of human anti- human immunodeficiency virus type 1 envelope
single-chain antibodies from a peripheral blood cell-based
phage repertoire

*Hans J.W. de Haard, Bert Kazemier, Peter Oudshoorn, Piet Boender,
Bob van Gemen, Marck J. M. Koolen, Guido van der Groen,
Hennie R.J.M. Hoogenboom and Jan-Willem Arends*

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1. Abstract

Monoclonal antibodies play an important role in the development of diagnostic assays. Instead of using hybridoma technology to isolate human immunodeficiency virus type 1-specific antibodies, a phage-displayed antibody library was generated from a small number (10^7) of peripheral blood lymphocytes from a seropositive donor. Two families of single-chain antibodies (scFvs) were selected by biopanning with the envelope precursor gp160. ELISA and competition in the BIAcore system revealed that one antibody family recognized a conformation-sensitive epitope within gp120, while the other antibody family was gp41-specific. The latter group had sequence similarity to antibodies recognizing the cluster III epitope of gp41. Binding of scFvs to gp160 could be inhibited with the donor's serum antibodies, indicating that antibodies with a similar specificity were circulating in the donor's blood. Competition experiments suggested that the epitope of the anti-gp41 antibodies was recognized by a broad range of patients' sera: 21 out of 22 sera from North American and all 20 sera from African seropositive patients inhibited binding of scFvs. In contrast, three sera from this panel did not react with the epitope of the anti-gp120 antibodies. These data indicate that, because of the conserved nature of its epitope, the anti-gp41 antibody will be suitable for diagnostic applications.

2. Introduction

Diagnosis of human immunodeficiency virus (HIV) infection by *in vitro* assays is performed by the detection of virus-specific antibodies or by detection of the virus itself, by means of associated antigens or by its genome and transcribed genes. Virus directed antibodies cannot be detected with currently used assays until 6 to 8 weeks after infection; detection of antigen in immunoassays or viral genes in nucleic acid-based tests shortens this so-called 'window' phase (Busch & Alter, 1995). Due to their high specificity, monoclonal antibodies are used for the capture and detection of antigens. These antibodies should be of high affinity and be directed against a conserved epitope. The affinity, which is directly related to the sensitivity of the assay (Lew, 1984; Devey & Steward, 1988), will determine the earliest time point of diagnosis. Also, the epitope of the antibody must be conserved, since the heterogeneity of the virus and its high mutation rate may lead to the complete failure of assays, as was recently found with sera from individuals infected with the subtype O virus (Gurtler, 1996).

In addition to diagnosis of infection, immunoassays could be used for monitoring therapy. For instance, during treatment of patients with nucleoside reverse transcriptase inhibitors or protease inhibitors alone (Spector *et al.*, 1989; Fischl *et al.*, 1990; Volberding *et al.*, 1990) or in combination (Harindra, 1997), replication of the virus is currently followed by CD4 counts and

the measurement of virus load by amplification or hybridisation methods. However, HIV treatment budgets are under considerable pressure. Therefore, a very sensitive immunoassay (Boni *et al.*, 1997) for the detection of the capsid protein (Von Sydow *et al.*, 1988; Graziosi *et al.*, 1993; Baumberger *et al.*, 1993) would be an attractive and cheap alternative to more expensive molecular diagnostics. When resistance occurs, demonstrated by a re-activation of the virus, treatment can be changed to another combination of drugs.

Immunoassays may also be used for prognosis and treatment strategy planning. The quantification of anti-gp41 and anti-p24 antibodies aids in the determination of the stage of the disease. During HIV pathogenesis, the titre of anti-gp41 antibodies remains more or less stable, while the titre of α -p24 antibodies gradually declines. Therefore, the ratio between these titres has prognostic value and can determine the appropriate treatment (Schmidt *et al.*, 1989; Strathdee *et al.*, 1995; Binley *et al.*, 1997). To allow quantification, monoclonal antibodies directed against p24 have been labelled with a reporter enzyme and used to compete with serum antibodies for antigen binding (Janvier *et al.*, 1991).

In this study we have evaluated phage antibody library technology (reviewed in de Haard *et al.*, 1998a) as a substitute for the traditional hybridoma technology. Theoretically, there are several advantages in applying phage-display technology, and patient-derived human antibodies in particular, to the generation of diagnostic reagents. Firstly, the recognition of immunodominant epitopes is crucial when antibodies are used in antibody-quantification assays. The immunization of mice does not necessarily result in the isolation of monoclonal antibodies which share the immunodominant epitopes recognized by human antibodies. Moreover, the presentation of the viral antigens during immunization might be different from the situation during infection, leading to the isolation of antibodies with different recognition patterns. Secondly, the conserved nature of the epitope is especially important in antigen detection. The existence of HIV variants and the mutational drift of the virus, which occurs in response to the appearance of neutralizing antibodies during infection (Watkins *et al.*, 1996), causes a high variability in all virus genes and their encoded proteins. The use of antigens from different virus strains in the biopanning procedure might favour the selection of such neutralizing antibodies. Thirdly, the application of human antibodies in antigen assays may avoid false-positive reactions caused by sera with human anti-mouse reactivity (HAMA); these sera were found sporadically during the development of a p24-antigen assay. Use of human scFv-fragments would even prevent problems with human anti-human responses (HAHA). Finally, the availability of the V genes permits the manipulation of antibody affinity (reviewed by Hoogenboom, 1997).

In this study we set out to generate human recombinant antibodies reactive against HIV-1, aiming mainly at their application as diagnostic reagents. As a source of antibody producing cells, a small number of circulating lymphocytes from a seropositive individual were used for the construction of the immune library. Biopanning was performed with the envelope precursor glycoprotein gp160, which is processed into the exterior envelope protein gp120 and the

transmembrane glycoprotein gp41. In addition to a family of gp120-specific antibodies, we selected a group of gp41-specific antibodies with a similar sequence to the cluster III epitope-recognizing antibodies isolated by Binley *et al.* (1997). The potential for both families of scFvs as diagnostic reagents was examined by competition with a panel of patients' sera, that could be expected to be diverse with respect to virus subtype. Only one serum, which gave unreliable results in other HIV-1 assays, did not recognize the gp41 epitope, while three sera failed to react with the gp120-epitope. Therefore, the anti-gp41 scFv seems to be the most promising candidate for application in *in vitro* diagnostics.

3. Materials and methods

3.1 Library construction and selections

Blood cells from a seropositive individual were separated on Ficoll Hipaque (Pharmacia) and stored in aliquots of 10^7 cells per vial in liquid nitrogen. For RNA isolation the cells from one vial were pelleted and washed with physiological salt solution. Approximately 2 μ g total RNA was extracted (Chirgwin *et al.*, 1979). One μ g was reverse transcribed into random-primed cDNA. IgG-specific variable heavy (VH) chain fragments were amplified using Amplitaq polymerase (Perkin-Elmer) for 30 cycles (94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 2 min) with HuVHBACK and HuIgG1-4CH1FOR primers (for primer sequences see Marks *et al.*, 1991). We combined the highly similar primers VH1a, VH3a and VH5a into one pool, VH4a and VH6a into a second group, and kept VH2a separately. In this way three different heavy chain amplification mixtures were obtained. The kappa light chain variable (VK) domains were amplified with the HuVKBACK and HuCKFOR primers. Primers VK1a and VK4a were combined, VK2a, VK3a and VK6a formed a second group, and VK5a was kept separate. The three heavy chain and three light chain fragments were isolated from a 1.5% agarose gel with the QIAex kit (QIAGEN) and reamplified with the corresponding J primers (HuJH1 and JH2 combined with JH4 and JH5, HuJH3 with JH6, HuJK1 combined with JK2, JK3 and JK4, while JK5 formed a separate group). We assembled the six heavy and six light chain fragments in PCR (Horton *et al.*, 1989) with the corresponding linkers into 36 fusion products. These were gel-purified, digested with *Sfi*I and *Not*I (Pharmacia), and cloned into the vector pV1, which is identical to pHEN1 (Hoogenboom *et al.*, 1991) except for the substitution of a FLAG-tag (Hopp *et al.*, 1988) for the Myc-tag (Evan *et al.*, 1985). Electroporation into *E. coli* JM101 was performed as described elsewhere (Dower *et al.*, 1988). To establish the degree of variation of the library, a *Bst*NI fingerprint (New England Biolabs) was performed on the PCR products of 24 individual clones (Marks *et al.*, 1991). All gave unique patterns, suggesting that no over-represented fragments were amplified and cloned.

The rescue of phagemid particles was performed as in Marks *et al.* (1991) except that for panning, 100 μ l 0.45 μ M-filtered culture supernatant (approx. 3×10^{10} transforming units) was

diluted with 900 µl sample diluent (PBS, 20% normal goat serum, 1.0% Triton X-100). The library was selected with the envelope precursor gp160, derived from the HIV-1 subtype III_B-secreting cell line 6D5₄₅₁ (Kalyanaraman *et al.*, 1988), and purified by affinity chromatography with an anti-gp41 monoclonal antibody as described before (Kalyanaraman *et al.*, 1990), and with the Gag-protein p24. The p24 antigen, expressed as full-length product in *E. coli* under control of the inducible *lac* promoter (constructed at Organon Teknika by M. Berman, unpublished results) and encoding the HIV-1 RF sequence, was affinity-purified with monoclonal antibody mAb 39B (generated at Organon Teknika, unpublished results). The proteins were coated as a mixture in 6-well tissue culture plates (Costar), at a concentration of 2.5 µg/ml for gp160 and 3 µg/ml for p24, in 50 mM Na₂CO₃ pH 9.6 for 16 h at 4 °C.

3.2 Screening and sequencing of clones

Soluble scFv was produced from individual clones by growth in V-shaped wells of microtitre plates, as described before (Marks *et al.*, 1991). For ELISA, microtitre plates were coated with 3 µg/ml p24, 2.5 µg/ml gp160 or 5 µg/ml virus lysate, in 50 mM Na₂CO₃ pH 9.6 for 16 h at 4 °C. The plates were blocked for 2 h with 0.2 % (w/v) BSA in 0.1 M Tris-HCl pH 7.4 plus 30 mM KI. Culture supernatants and periplasmic fractions were diluted in sample diluent. After incubation at room temperature for 2 h, bound scFv was detected with anti-FLAG antibody M2 (Kodak IBI), diluted 1:4000 in sample diluent. After a 1 h incubation with 1:2000 diluted anti-mouse IgG-horseradish peroxidase conjugate (anti-mouse-HRP, DAKO), staining was performed for 30 min with tetramethylbenzidine (TMB) and urea peroxide (Organon Teknika) as substrates. The reaction was stopped by adding an equal volume 1M H₂SO₄; the absorbance was measured at 450 nm.

Clones giving positive signals and with a unique *Bst*NI fingerprint were analysed by sequencing using the T7 sequencing kit (Pharmacia) with the M13rev primer (5' CAGGAAACAGCTATGAC 3'), the gene III primer (5' TGAATTTTCTGTATGAGG 3') and two primers located in the linker (LINKFWD, 5' CTCGGGCGGTGGTGGGTC 3'; LINKREV, 5' GACCCACCACCGCCCGAG 3'). Plasmid DNA was purified with the QIAgen kit (QIAgen).

3.3 Characterization of scFvs

Periplasmic fractions were prepared with borate-buffered saline (BBS) (Skerra & Pluckthun, 1991). The cells from a 50 ml culture were resuspended in 1 ml BBS (200 mM boric acid-NaOH pH8.0, 160 mM NaCl). After 2 to 16 h incubation at 4°C, spheroplasts were pelleted by a differential centrifugation step: 10 min at 6000 g in an Eppendorf centrifuge followed by an additional clearance of the supernatant at 13000 g for 30 min.

Virus lysate, BSA and gp160 were loaded onto 12% SDS-PAGE gels and blotted onto nitrocellulose. After blocking with a skimmed milk solution (5% w/v, in TBS), periplasmic fractions were added to the solution (10% v/v). Bound scFv was detected subsequently with anti-

FLAG M2, diluted 1:4000 in TBS plus Triton X-100, and with anti-mouse alkaline phosphatase (anti-mouse-AP, Promega), diluted 1:4000 in TBS.

The specificity of the scFvs was examined by ELISA with immunopurified gp120 (Kalyanaraman *et al.*, 1990), a constrained V3 loop with the sequence CTRPNNNTRKSIHIGPGRAFYYTTGEIIGDIRQAHC (Tolman *et al.*, 1993) and a constrained gp41-derived peptide with the sequence RILAVEERYLKDQQLLGtWGCSGKLIC (Gnann *et al.*, 1987) as antigens.

For competition ELISA, plates were coated with gp160 as described above. For inhibition with serum antibodies from the donor of the peripheral blood lymphocytes (PBL) used for library construction, a fixed concentration of scFv from clones AB#31 and AB#32 was applied (1:1000- and 1:750-diluted periplasmic fraction, respectively), while the serum was diluted 1:100, 1:1000 and 1:10000. For competition with heterologous sera, a panel was tested containing samples from 22 North American (Mexico and USA) and 20 African (Cameroon, Ivory Coast and Tanzania) HIV-1 infected individuals. The scFvs, diluted as above, and the serum, 50-fold diluted, were incubated simultaneously; scFv bound to gp160 was detected with α -FLAG and anti-mouse-HRP.

Epitope mapping was performed with BIAcore (Malmborg & Borrebaeck, 1995). gp160 was coupled covalently to a CM-chip (BIAcore AB) according to the supplier's instructions. The different classes of gp160-binding scFv were injected sequentially, yielding equilibrium signals. Antibody fragments recognizing different epitopes should give additional responses, whereas an unchanged signal suggests the recognition of a common epitope.

Affinities were determined by kinetic analysis with surface plasmon resonance (BIAcore) using a CM5-chip containing 2856 response units (RU) immobilized gp160. Three and four different concentrations of scFv were injected for AB#31 and AB#32, respectively. The on-rate was calculated at the concentration of scFv present in periplasmic fractions, which was determined by densitometric measurement of anti-FLAG M2-incubated Western blots, with a purified murine anti-chorionic gonadotrophin scFv as a standard (see also de Haard *et al.*, 1998c).

4. Results

4.1 Library construction and panning

For the generation of a random combinatorial library, we extracted RNA from PBL of a seropositive individual. The donor, a haemophilia sufferer, was infected with HIV-1 in 1981 by a blood-derived coagulation product originating in the USA. The patient's seroconversion was demonstrated by Western blot (Fig. 1): a response against the virus core derivatives emerged 23 months before the PBLs were collected (lane 2). Seven months before harvesting, antibodies

directed against the envelope derivatives gp120 and gp41 and polymerase derivatives p64, p51 and p31 were present (lane 3). A sample of 10^7 lymphocytes that had been stored in liquid nitrogen for 10 years was used for RNA isolation. The VH and VK chains were amplified and assembled with the linker-encoding DNA fragment into the scFv gene segments. The library obtained consisted of 5×10^7 clones, of which 97% had a complete scFv gene (not shown).

The library was subjected to four rounds of panning with a combination coat of gp160 and p24. This selection produced a 5000-fold enrichment in the number of eluted phage particles, relative to the first round. The fraction of phage carrying a complete scFv-encoding insert in the genome was determined by colony morphology (de Haard *et al.*, 1998b): it increased from 60% after the first round to 98% after the last round. The polyclonal phage amplification products, which were harvested before each panning round, were tested by ELISA on the combination coat of gp160 and p24 and also on gp160 alone: both assays gave increasing signals after the second round (data not shown). Twenty-four individual clones picked randomly from the third round and 24 clones from the fourth round were grown in a microtitre plate. The scFvs that leaked into the culture supernatant gave positive reactions against both the combination coat and gp160 alone and were negative against p24 and BSA. *Bst*NI-fingerprinting revealed the presence of two different classes of clones. One clone from each of the two classes was analysed in more detail; these were designated AB#31 and AB#32.

Fig. 1. Analysis of the response of serum from the PBL donor against HIV-1 antigens. Only one band was visible on the strip incubated with pre-infection serum (taken on 1 October 1981, lane 1), which is a cellular component present in the virus lysate. In lane 2, incubated with serum taken on 1 May 1982, the characteristics of an early response are visible, i.e. a predominant reactivity against the core derivatives p55, p24 and p17. Lane 3, incubated with a serum sample collected on 23 September 1983, reveals the late response directed mainly against the envelope derivatives gp160 and gp41 and the polymerase products p64, p51 and p31.

We repeated the panning procedure with gp160 alone, and found similar degrees of enrichment as when using the combination of antigens. *Bst*NI fingerprints of clones were obtained for antibody fragments that gave positive signals in ELISA; the similar *Bst*NI fingerprints suggested that the clones were closely related to those obtained in previous panning experiment. We examined five new clones, designated #B4, #B5, #B7, #D1 and #D4, in more detail.

4.2 Sequence analysis

The sequences of the gp160-binding antibody fragments selected from the human library are shown in Fig. 2. Selection on gp160 yielded two different types of antibodies with heavily mutated VH genes. Comparison with the germline sequences (Tomlinson *et al.*, 1992) shows that clone AB#31 and its derivatives #B5 and #B7 use a VH1 family-derived germline segment (DP-7). Clone AB#31 has 30 differences from the germline analogue within its nucleotide sequence, leading to 15 amino acid substitutions. In contrast, clone #32 and its derivatives #B4, #D1 and #D4 use a germline segment of the VH3 family (coded DP-46): for clone AB#32 there are only 17 differences in its nucleotide sequence resulting in 12 different amino acids. Alignment with the V κ germline sequences (Cox *et al.*, 1994) showed that both types of gp160-specific antibodies use the same segment (V κ 3 family; coded DPK22/A27) although joined to different J κ segments. The V κ segments are less mutated: only three and seven differences in nucleotide sequence resulting in one and three amino acid substitutions, were observed for clones AB#32 and AB#31, respectively.

The use of one type of V κ for both antibodies might indicate a low complexity of the amplified V κ -fragments, but a closer examination of the sequences reveals the presence of subtype-specific residues resulting from somatic hypermutation. This can be seen with residue 77 (Fig. 2) and with the J segment-encoded residue at position 96. Ditzel *et al.* (1997) found a light chain nearly identical to the one used by AB#31 and AB#32, but paired with different heavy chain fragments, in their anti-gp120 antigen-binding fragment (Fab).

The heavy chain CDR3 region of the anti-gp120 antibodies is rather long (20 residues), while the one from the anti-gp41 antibody is much shorter. The existence of somatic hypermutation can be identified in the heavy chain sequences of the AB#32 family. The ancestor VH segment, which is found in clone #B4, contains three germline residues, at position 26, 30 and 56, that are all mutated in the other members of the family (Fig. 2). A similar phenomenon is

Fig. 2 (on next page). Amino acid sequences of the VH and VL chain regions of the anti-gp160 antibodies. Numbering of residues and classification of heavy and light chains follows Kabat *et al.* (1991). Primer-encoded differences within the amino- and carboxy-terminal regions are indicated with lower-case characters. The variable region sequences of the AB#31 (anti-gp41) and AB#32 (anti-gp120) derivatives are grouped separately with the corresponding germline V genes. The most similar germline VH segments are DP-7 for AB#31/#B7/#B5 and DP-46 for AB#32/#D4/#B4/#D1. For VL the germline segment DPK22/A27 was used in all clones. HS3D6HCV-1 represents the published light chain sequence of an anti-gp41 antibody and p35 indicates a phage library-derived anti-gp120 Fab.

[illegible]

seen in the heavy chain of the AB#31 family: the germline residues 65 and 69 in clone #B7 have been mutated in clone AB#31. Finally, comparison of the sequence of the V κ region from clones #B6 and #B7 with AB#31 showed that the first two contain ten additional hypermutated residues. At present we do not know if any of these mutations lead to an increase in affinity.

In a comparison of the amino acid sequence of the VH regions using the BLAST program, a similarity was found between the scFv from AB#32 with a human monoclonal antibody, coded HS3D6HCV-1 (Fig. 2), directed against gp41 (Felgenhauer *et al.*, 1990). A greater homology existed between the V κ sequences of AB#31/AB#32 and the phage library-derived anti-gp120 Fabs p35 and p20 (Ditzel *et al.*, 1997) (see Fig. 2). The heavy chain sequences of p20 and p35 do not resemble those of scFv from AB#31 and AB#32, although they share a long CDR3 region (17 residues for p20 and p35 compared with 20 residues for AB#32) with AB#32.

Fig. 3. Epitope mapping with the scFv fragments from clones AB#31 and AB#32 on the BIAcore with gp160 coupled to a CM5-chip at a density of 2856 RU.

Panel IA. After 50 s the periplasmic fraction of clone AB#31 was injected, until at 640 s the Hepes Buffered Saline-wash started. The periplasmic fraction of clone #32 was injected from 800 s to 1350 s.

Panel IB. Overlay of the curves from AB#31 with a prolonged dissociation phase and from AB#32 [obtained from panel IIB]. The curve of AB#32 was superimposed at 800 s on the level for AB#31 at 1350 s. In this way the loss in signal due to dissociation of AB#31 can be corrected, resulting in the observed end-level.

Panel IIA. Sequential injection of AB#32 and AB#31 was carried out at the same time points as in (IA). Because of the lower dissociation rate of AB#32, the increased signal caused by AB#31 can be recognized more easily. The end-level at 1500 s (450 RU) is identical to that found in panel IA, showing that the signals are additive.

Panel IIB. Overlay of the curve obtained from AB#32 with a prolonged dissociation phase, superimposed at 800 s on that obtained for AB#31 at 1350 s [obtained from IB], confirming additivity.

4.3 Characterization of scFvs

The specificity of both types of antibodies was analysed with BIAcore (Malmborg & Borrebaeck, 1995). The scFv-containing periplasmic fractions were injected sequentially onto a gp160 surface (Fig. 3). When AB#32 was injected first, an additional signal was obtained from the subsequent injection of AB#31 (Fig. 3, panel IIA), suggesting that the antibodies recognize distinct epitopes on gp160. The total signal obtained from both antibodies (at 1500 s the response was 450 RU) can be interpreted as the sum of the signals from both scFvs alone [the response of AB#32 at 1500 s was 130 RU (panel IIB) and that of AB#31 at 800 s was 320 RU (panel IA)]. Additivity can be demonstrated by overlaying the individual binding curves (Fig. 3 panel IIB).

The signal obtained by injection of AB#31 followed by AB#32 (Fig. 3 panel IA) seems not to be additive, but this appearance is caused by the somewhat faster dissociation of AB#31. The curve obtained from AB#31 with a prolonged dissociation phase (Fig. 3, panel IB) shows that the total signal at 1500 s (430 RU) can also be considered as the sum of the signals of the individual antibodies: at 1500 s the signal of AB#31 was 280 RU (Fig. 3, panel IB) and at 800 s the signal of AB#32 was 150 RU (Fig. 3, panel IIA).

The affinities of scFv AB#31 and AB#32, determined by kinetic analysis of association and dissociation with surface plasmon resonance (Table 1), are in the nanomolar range. AB#32 has a 12-fold higher affinity than AB#31, resulting from a 1.4-fold slower dissociation rate and a 8.6-fold faster association rate.

Table 1. Affinities of anti-HIV-1 envelope scFvs

Clone	k_{off} (sec^{-1})	k_{on} ($\text{M}^{-1}.\text{sec}^{-1}$)	K_d (M)
AB#31	$(1.88 \pm 0.43) \times 10^{-3}$	$(5.38 \pm 0.77) \times 10^5$	$(3.49 \pm 1.29) \times 10^{-9}$
AB#32	$(1.33 \pm 0.10) \times 10^{-3}$	$(4.64 \pm 1.10) \times 10^5$	$(2.87 \pm 0.89) \times 10^{-10}$

AB#31 and AB#32 were used for the detection of HIV antigens on an immunoblot (Fig. 4). Only the non-reduced forms of the envelope protein gp120 and its precursor gp160, which is actually a truncated form missing the 68 carboxy-terminal amino acids, were recognized by AB#32 [both antigens are marked in Fig. 4A]. AB#31 was specific for gp160 and not for gp120, although the signal on the blot was less intense than that obtained with AB#32. Omitting boiling of the virus lysate before loading on the gel improved the sensitivity of detection: a more intense gp160, but also a faint gp41 band, were visible on the blot incubated with AB#31 (Fig. 4B). The bands showing up in the gp120 preparation are derived from the mouse antibody used for purification, which leaked from the affinity column; it was also detected with the anti-mouse conjugate [see left blot of Fig. 4A]. The proteins reactive with AB#32 with molecular masses

greater than 200 kDa are the dimeric products of gp120 and gp160, which were also visible on a blot stained with amido black. In conclusion, AB#31 recognizes a conformation-sensitive epitope of gp160, that is probably located in the transmembrane glycoprotein gp41, and AB#32 reacts with a conformation-sensitive epitope that is present in gp120.

Fig. 4. Analysis of the response of the recombinant antibodies against HIV-1 antigens on a non-reducing Western blot.

(A) Blots were incubated with either anti-mouse-AP (α -Mo), AB#31 or AB#32, and the marker lane (M) was stained with Ponceau S. Viral lysate (about 50 μ g), purified gp160 (2 μ g) and gp120 (2 μ g) were loaded. The positions of gp160 and gp120 are indicated.

(B) Detailed analysis of reactivity of AB#31 and AB#32. Virus lysate and gp120 samples were boiled (+T) or loaded directly on the gel without heat treatment (-T). gp41 was detected by AB#31 and not by AB#32.

The specificities of the scFvs from AB#31 and AB#32 were analyzed further by ELISA. Immunopurified gp120, a constrained V3 loop peptide of gp120 (Tolman *et al.*, 1993) and a constrained peptide of gp41 (Gnann *et al.*, 1987), all based on the HIV-1 sequence, were used to

coat microtitre plates. A response was only observed for AB#32 against gp120. Therefore, the epitope of AB#31 must be located within gp41, and it differs from the Gnann epitope. The epitope of AB#32 resides in gp120, but it is not located within the immunodominant V3 loop.

Fig. 5. Inhibition of binding of AB#31 (A) and AB#32 (B) to gp160 in ELISA by serum antibodies from the donor of the PBL, that were used for the construction of the library. Pre-infection serum (taken on 19 August 1980, tinted bars) and post-infection serum (taken on 17 September 1983) were diluted 100-fold (-2), 1000-fold (-3) and 10000-fold (-4). Results are expressed relative to those obtained with scFv without adding serum.

We performed an inhibition ELISA in order to establish the presence of the isolated antibodies in the serum of the donor. The patient's serum, taken before and after HIV infection, the latter with anti-gp160 reactivity, was mixed with the scFvs and incubated in wells coated with gp160. Subsequently, bound scFv was detected by its FLAG-tag. As is shown in Fig. 5, the binding of AB#31 to the antigen was almost completely inhibited by the serum antibodies: 94.7% inhibition was observed with a 100-fold diluted serum and 29.0% at a 10000-fold dilution. Binding of AB#32 was also competitively inhibited, but to a lesser extent: 76.0% inhibition was observed at a 100-fold dilution. In contrast, the pre-infection serum did not affect binding of the recombinant antibodies to gp160. This experiment shows that antibodies sharing the epitopes with the library-derived scFvs were present in the serum of the donor. The antibody

fragments are therefore not artificial products with unusual specificities resulting from the scrambling of VH and VL genes during library assembly.

Fig. 6. Competition for binding to gp160 in ELISA between AB#31 (above) or AB#32 (below) and heterologous sera. Three sera did not contain antibodies that competed for binding with AB#32 (inhibition <20%), while antibodies from all but one serum competed for binding with AB#31. Binding by AB#31 (mean inhibition 95.5%, SD 3.0%) was competed more efficiently than that by AB#32 (African samples, $69.9 \pm 19.9\%$; North American samples, $86.3 \pm 13.6\%$).

4.4 Competition with heterologous sera

To explore the possible application of the scFvs as diagnostic reagents, we analysed the epitope recognition of the selected antibody fragments in more detail. A competition assay was set up, in which a panel of anti-gp160 positive sera from 22 North American (USA and Mexico) and 20 African (Cameroon, Ivory Coast and Tanzania) patients was tested; the results obtained are shown in Fig. 6. One serum sample from a North American patient showed a low degree of inhibition (8.4%) of AB#31 binding. The other sera competed well with AB#31 for binding to

gp160: mean inhibition was 95.5% with an SD of 1.5%. For AB#32 the picture was different. The mean percentage inhibition was much lower (69.9% with an SD of 19.9%), and serum from three North American patients, including the one that did not compete with AB#31, did not compete for binding. The serum that did not inhibit binding of either scFvs to gp160 was negative on an immunoblot for binding to p18, p24, p31 and gp41, indeterminate for p51, p55, p65 and gp120 and positive for gp160. The indeterminate and the negative results against gp120 and gp41, respectively, suggest that the signal against gp160 might be caused by non-specific interactions. This serum gave a borderline signal when tested in the Clonatec assay, which contains a peptide with an immunodominant epitope of gp41. The difference between AB#31 and AB#32 in their sensitivity for competition can partly be explained by their affinities: binding of AB#32 to gp41, with a 12-fold lower K_d than that of AB#31 (Table 1), might be inhibited inefficiently.

We conclude that the epitopes of AB#31 and AB#32 are recognized by the serum antibodies from North American and African seropositive patients. This implies that both recombinant antibodies recognize conserved epitopes, thereby making them suitable candidates for application in *in vitro* diagnostics.

5. Discussion

5.1 Small numbers of PBL as a source for library construction

Combinatorial library technology has already been applied to the generation of human Fabs directed against the HIV-1 envelope (Burton *et al.*, 1991; Barbas *et al.*, 1993; Binley *et al.*, 1997). We selected antibodies from PBL from a haemophilia sufferer (since deceased) who was infected with hepatitis C virus, and later with HIV-1, through administration of blood-derived coagulation products, and who had no acute immune response at the time of collection of the blood cells. RNA was extracted from a small number of cells (10^7). In spite of the poor condition of the cells, as deduced from their low viability, we managed to isolate a small amount of RNA. After panning of the human library, two types of gp160-specific antibodies were selected, which have specificities matching serum antibodies from the donor. The two types have different sequences (VH1 and VH3 families), and recognize different epitopes of gp160. The successful selection of these antibodies from a PBL library was surprising, since it has been suggested that the preferred source of antibody-producing B cells is bone marrow (reviewed by Parren & Burton, 1997). It has been reported that the selection of anti-tetanus toxoid Fabs from PBL libraries was possible from recently boosted donors (Mullinax *et al.*, 1990; Persson *et al.*, 1991), but not from individuals who had not been recently immunized, in spite of the presence of high levels of circulating anti-tetanus toxoid antibodies in their blood (Persson *et al.*, 1991). Most of the circulating lymphocytes are memory cells expressing small amounts of antibody, transcribing

up to 100 copies of Ig-encoding mRNA per cell, in contrast to plasma cells, which synthesize 30000 copies of Ig mRNA (Schibler *et al.*, 1978). We demonstrated that seroconversion occurred in our donor approximately 30 months before the lymphocytes were collected. One year before collection, the serum contained IgG against gp120, gp41, p24 and other antigens, as was detected by Western blot. These data suggest that at the time the PBL were harvested the donor did not have an acute response, in which elevated numbers of circulating plasma cells can be expected.

In spite of the presence of anti-p24 antibodies in the donor's serum, we did not retrieve such antibodies from the library during panning with the combination of gp160 and p24. This might be explained by the low complexity of the library, resulting from the small amount of RNA used for its construction, but it might also be caused by the competitive nature of panning. We believe that the outcome of selection on a mixture of antigens is the antibody with the highest affinity or antibodies with comparable affinities for one of the components, even for the protein used for blocking. This effect has been demonstrated before (Ditzel *et al.*, 1995), during the selection of gp120-specific Fabs. The only way of selecting antibodies that were not reactive against the CD4-binding domain was to shield this epitope through binding to an antibody. Another reason for the absence of anti-p24 scFvs might be a decreasing titre of anti-p24 antibodies in the patient, as is often seen during HIV-1 pathogenesis (Forster *et al.*, 1987; Chargelegue *et al.*, 1993).

The anti-gp120 scFv AB#32, with a VH3 germline segment, contains a relatively long heavy chain CDR3 regions (20 residues). The library derived anti-gp120 Fab isolated by Barbas and colleagues (Barbas *et al.*, 1993) contained VH1 as well as VH3 segments, with the CDR3-length varying from 18 to 22 residues. No significant similarity was apparent with the VH sequence of our anti-gp120 scFvs. However, there is a most remarkable similarity between the V κ sequences of two recently isolated anti-gp120 Fabs (Ditzel *et al.*, 1997) and those of our clones. The light chains of the published Fabs were fused alternatively to the JK segments, leading to a CDR3 of 9 and 10 residues in clone p35 and p20, respectively, and they paired with different heavy chains, as was also observed for AB#31 and AB#32.

5.2 Possible diagnostic applications

Our main interest is the application of monoclonal antibodies to *in vitro* diagnostics. The use of antibodies of human origin in antigen assays prevents false positive reactions caused by sera with HAMA-reactivity. Moreover, the epitopes involved must be conserved and recognized by a broad range of patient's sera. Immunization of mice does not necessarily lead to the isolation of monoclonal antibodies which react with these kinds of epitopes.

The use of the BIAcore system revealed that the selected scFvs react with distinct epitopes on gp160, as was confirmed by ELISA against gp120. AB#32 reacts with a conformation-sensitive epitope of gp120 different from the V3 loop. In contrast, Western blot analysis with scFv as the detection antibody suggested that AB#31 has gp41-specificity, explaining the

absence of reactivity of this antibody against gp120 in ELISA and immunoblot. The epitope recognized by this scFv does not coincide with the immunodominant 'Gnann epitope' (Gnann *et al.*, 1987), which is part of the cluster I epitope on gp41. According to the sequence of its VH chain, AB#31 appears to be related to the cluster III epitope-recognizing recombinant Fabs described by Binley *et al.* (1997). Antibodies to this cluster use the same germline VH gene (DP-7) and share the CDR3 length (12 residues). A faint similarity can be found between AB#31 and the cluster III clone G15 in the CDR3-encoded [AB#31, PFMNQLRNAID; G15, PRFNLIRE(A/P)LDL], and both use a V κ 3 light chain gene. Comparison of clone AB#31 with G15 revealed 36 differences in the deduced amino acid sequence of VH, but 20 of the differing residues could also be found in one or more of the other cluster III clones G5, L11, and in particular in clones L1 and A9 (data not shown). The occurrence of identical somatically mutated residues within CDR1 (residue 31 and 32) and CDR2 (residue 65) in the cluster III clones and clone AB#31 strengthens the hypothesis of a relation between these antibodies. These data suggest that AB#31, which like the cluster III Fabs of Binley *et al.* (1997) only reacts with native antigen on an immunoblot, recognizes a conformational epitope involving residues 619-648 and probably residues 563-583 of gp41 (for numbering see Binley *et al.*, 1997).

Inhibition ELISA with a broad range of anti-gp160-positive sera from seropositive individuals, not only from North America, but also from the Africa, where variability among HIV-1 viruses is much larger than elsewhere (Nkengasong *et al.*, 1994; Louwagie *et al.*, 1995), showed that the epitope on gp41 is immunodominant and conserved. The presence of immunodominant and conserved epitopes on gp41 is well documented (Gnann *et al.*, 1987; Cumming *et al.*, 1990; Muster *et al.*, 1993), and confirms the idea that AB#31 in particular, which is reactive against this antigen, will be useful for diagnostics.

Besides conservation of its recognized epitope, another important feature of a diagnostic antibody is affinity, since this will determine the sensitivity of the assay (Lew, 1984; Devey & Steward, 1988). Kinetic measurements with surface plasmon resonance revealed high affinities for both antibodies: AB#31 has an affinity in the nanomolar range (3.5 nM), while AB#32 has even greater affinity (0.29 nM),

One diagnostic application of these scFvs could be the quantification of anti-gp41 antibodies during HIV-1 pathogenesis. The ratio of anti-p24 to anti-gp41 antibodies has prognostic value: the titre of anti-p24 gradually declines, whereas the anti-gp41 titre remains unchanged (Schmidt *et al.*, 1989; Strathdee *et al.*, 1995; Binley *et al.*, 1997). A quantitative assay for anti-gp41 antibodies could be made by construction of labelled scFv AB#31 that could be used for the competition of serum antibody-binding to immobilized antigen, as has been described before for p24 (Janvier *et al.*, 1991). The format of the scFv can be engineered for this purpose, as has been demonstrated previously (Kerschbaumer *et al.*, 1997).

6. References

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CHAPTER 7

ANTI- GLYCOPROTEIN HORMONE FAB'S FROM A NAIVE HUMAN REPERTOIRE

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A large naïve human Fab fragment phage library that permits rapid
isolation and kinetic analysis of high affinity antibodies

*Hans J.W. de Haard, Nicole van Neer, Anneke Reurs, Simon E. Hufton,
Rob C. Roovers, Paula Henderikx, Jan-Willem Arends,
and Hennie R.J.M. Hoogenboom*

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1. Abstract

We report the design, construction and use of the first very large naïve phage antibody library in Fab format, that allows the rapid isolation and affinity-analysis of antigen-specific human antibody fragments. Individually cloned heavy and light chain variable region libraries were combined in an efficient 2-step cloning procedure, permitting the cloning of in total 3.7×10^{10} independent Fab clones. The performance of the library was determined by the successful selection of on average 14 different Fab's against 6 antigens tested. These include tetanus toxoid, the hapten phenyl-oxazolone, the breast cancer associated MUC1 antigen and three highly related glycoprotein hormones: human Chorionic Gonadotropin, human Luteinizing Hormone and human Follicle Stimulating Hormone. In the latter category, a panel of either hormone-specific or cross-reactive antibodies were identified. The design of the library permits the monitoring of selections with polyclonal phage preparations and to carry out large scale screening of antibody off-rates with unpurified Fab fragments on BIAcore. Antibodies with off-rates in the order of 10^{-2} to 10^{-4} s⁻¹ and affinities up to 2.7 nM, were recovered. The kinetics of these phage antibodies are of the same order of magnitude as antibodies associated with a secondary immune response. This new phage antibody library is set to become a valuable source of antibodies to many different targets, and to play a vital role in target discovery and validation in the area of functional genomics.

2. Introduction

Display on filamentous phage in combination with selection forms a powerful tool for the identification of peptide- or protein-based drugs (1,2). Of these, antibodies are especially of interest, due to their capacity to recognize a variety of targets with high specificity and affinity. Particularly the use of partially or completely human antibodies, which elicit no or minimal immune response when administered to patients, is yielding an increasing list of FDA-approved protein-based drugs (3). Phage display technology enables the generation of large repertoires of human antibodies (4-7), while the biopanning procedure permits the selection of individual antibodies with a desired specificity.

Key to the success of the technology were two critical observations: (i) the expression of functional antibody fragments by secretion into the periplasm of *E. coli* (8,9), and, (ii) the rapid access to variable region gene pools by the polymerase chain reaction (10-12). For the construction of antibody libraries, V-genes are amplified from B-cell cDNA and heavy and light chain genes are randomly combined and cloned to encode a combinatorial library of single-chain Fv (scFv) or Fab antibody fragments (4,13-15). The natural primary (unselected) antibody

repertoire within B-cells contains a large array of antibodies that recognize a variety of antigens; this array can be cloned as a 'naïve' repertoire of rearranged genes, by harvesting the V-genes from the IgM mRNA of B-cells of unimmunized human donors, isolated from peripheral blood lymphocytes (4), bone marrow or tonsils (7), or from similar animal sources (16). This procedure provides access to antibodies that have not yet encountered antigen, although the frequency of those genuine 'germline' antibodies will depend heavily on the source of B-cells (17). A single 'naïve' library, if sufficiently large and diverse, can indeed be used to generate antibodies to a large panel of antigens, including self, non-immunogenic and relatively toxic antigens (6,18). In a different approach, antibodies may be built artificially, by in vitro assembly of V-gene segments and D/J segments, yielding 'synthetic' antibodies (5). A major drawback of these procedures is that from the initial 'naïve' and 'synthetic' libraries, only moderate affinity antibodies were isolated (18,19). Over the last few years more efficient techniques have been developed to build larger libraries of antibody fragments, using sophisticated in vivo recombination methods (6) or brute force cloning procedures (7,20). Such large libraries have yielded a greater number of human antibodies per antigen tested, with an average much higher affinity (up to sub-nanomolar). However, technical restrictions on the size of libraries that may be obtained or handled in selection, the loss of library diversity upon library amplification, and the relatively long down-stream analysis path of the selected antibodies, i.e. large scale affinity analysis, have limited the spread of these libraries as generic tools in antibody generation.

We describe here the strategy and construction of a very large phage antibody library, based in a phagemid vector, from which high-affinity human Fab fragments can be selected. The choice for the Fab format was based on the notion that the monomeric appearance of the Fab should permit the rapid screening of large numbers of clones on kinetics of binding (off-rate) with crude protein fractions. This would reduce the time for post-selection analysis dramatically when compared to that needed for selected single-chain Fv (scFv) antibodies from phagemid libraries (7,20), or Fab fragments from phage libraries (6). By applying an efficient cloning strategy, in which restriction fragments instead of PCR-products were used, we generated a repertoire with as many as 37 billion different Fab clones. The performance of the library was analysed by the selection with an extended panel of antigens including three closely related glycoprotein hormones, yielding a diverse set of specific antibody fragments for each antigen. Without using sophisticated selection protocols, hormone specific as well as cross-reactive Fab's were retrieved against the highly homologous glycohormones, demonstrating that the library is a rich source of antibody specificities. The affinities of the anti-glycohormone antibodies varied between 2.7 and 38 nM. Finally, the Fab-format indeed permitted the rapid screening and a reliable ranking of individual clones on off-rate using crude fractions.

3. Materials and methods

3.1 RNA-isolation

As source of lymphoid tissues we used peripheral blood lymphocytes from 4 healthy donors and part of a tumor-free spleen removed from a patient with gastric carcinoma. B lymphocytes were isolated from 2-L of blood on a Ficoll-Paque gradient. For RNA isolation, the cell pellet was immediately dissolved in 50 ml 8 M guanidinium thiocyanate / 0.1 M 2-mercaptoethanol (21). Chromosomal DNA was sheared to completion by passing through a narrow syringe (1.2/0.5 mm gauge), and insoluble debris was removed by low speed centrifugation (15 min 2,934xg at room temperature). RNA was pelleted by centrifugation through a CsCl-block gradient (12 ml supernatant on a layer of 3.5 ml 5.7 M CsCl / 0.1 M EDTA; in total 4 tubes) during 20 h at 125,000xg at 20°C in a SW41-rotor (Beckman). The yield of total RNA was approx. 600 µg. RNA was stored at -20°C in ethanol.

From the spleen, 2 g of tissue was used for homogenisation with a polytron in 20 ml 8 M guanidinium thiocyanate / 0.1 M 2-mercaptoethanol. The total volume was increased to 80 ml with guanidinium thiocyanate buffer, and after passage through a narrow syringe for shearing and removal of debris, RNA was pelleted as described before, except for 15 h at 85,000xg at 20°C in a SW28.1 rotor (12 ml supernatant on 3.5 ml 5.7 M CsCl / 0.1 M EDTA in 5 SW28.1 tubes). From 2 g of tissue, 3 mg of total RNA was extracted.

3.2 Amplification of variable region genes

Random primed cDNA was prepared with 250 µg PBL RNA, while in a separate reaction 300 µg spleen RNA was used as template. RNA was heat denatured for 5 min at 65°C in the presence of 20 µg random primer (Promega), subsequently buffer and DTT were added according to the suppliers instructions (Gibco-BRL), as well as 250 µM dNTP (Pharmacia), 800 U RNasin (40 U/µl; Promega) and 2,000 U MMLV-RT (200 U/µl; Gibco-BRL) in a total volume of 500 µl. After 2 h at 42°C, the incubation was stopped by a phenol / chloroform extraction; cDNA was precipitated and dissolved in 85 µl water.

Oligonucleotides used for PCR amplification of human heavy and light chain V-regions are described in Table I. IgM-derived heavy chain variable regions were obtained by a primary PCR with an IgM constant region primer. All primary PCRs were carried out with separate BACK primers and combined FOR primers, to maintain maximal diversity. The PCR-products were reamplified with a combination of JHFOR-primers, annealing to the 3' end of VH, and Sfi-tagged VHBACK-primers, annealing to the 5' end, and subsequently cloned as VH-fragments. The light chain V genes of the kappa and lambda families were obtained by PCR with a set of CKFOR- or λ FOR-primer annealing to the 3' end of the constant domain and BACK-primers, priming at the 5' end of the V-regions. The DNA-segments were reamplified with primers tagged with restriction sites and cloned as $V\kappa$ CK- and $V\lambda$ C λ -fragments.

Table I. Oligonucleotide primers used for construction of the library

A. Primary amplification		B. Secondary amplification	
IgM CH1 region			
HuIgMFOR	5'-TGG AAG AGG CAC GTT CTT TTC TTT-3'		
κ chain constant region		κ chain constant region	
HuCKFOR	5'-ACA CTC TCC CCT GTT GAA GCT CTT-3'	HuCK-FOR-ASC	5'-ACC GCC TCC ACC GGG CGC GCC TTA TTA ACA CTC TCC CCT GTT GAA GCT CTT-3'
λ chain constant region		λ chain constant region	
HuCλ2-FOR	5'-TGA ACA TTC TGT AGG GGC CAC TG-3'	HuCλ2-FOR-ASC	5'-ACC GCC TCC ACC GGG CGC GCC TTA TTA TGA ACA TTC TGT AGG GGC CAC TG-3'
HuCλ7-FOR	5'-AGA GCA TTC TGC AGG GGC CAC TG-3'	HuCλ7-FOR-ASC	5'-ACC GCC TCC ACC GGG CGC GCC TTA TTA AGA GCA TTC TGC AGG GGC CAC TG-3'
V _H back		V _H back	
HuVH1B/7A-BACK	5'-CAG RTG CAG CTG GTG CAR TCT GG-3'	HuVH1B/7A-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG RTG CAG CTG GTG CAR TCT GG-3'
HuVH1C-BACK	5'-SAG GTC CAG CTG GTR CAG TCT GG-3'	HuVH1C-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTR CAG TCT GG-3'
HuVH2B-BACK	5'-CAG RTC ACC TTG AAG GAG TCT GG-3'	HuVH2B-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG RTC ACC TTG AAG GAG TCT GG-3'
HuVH3B-BACK	5'-SAG GTG CAG CTG GTG GAG TCT GG-3'	HuVH3B-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH3C-BACK	5'-GAG GTG CAG CTG GTG GAG WCY GG-3'	HuVH3C-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG WCY GG-3'
HuVH4B-BACK	5'-CAG GTG CAG CTA CAG CAG TGG GG-3'	HuVH4B-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTA CAG CAG TGG GG-3'
HuVH4C-BACK	5'-CAG STG CAG CTG CAG GAG TCS GG-3'	HuVH4C-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG STG CAG CTG CAG GAG TCS GG-3'
HuVH5B-BACK	5'-GAR GTG CAG CTG GTG CAG TCT GG-3'	HuVH5B-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAR GTG CAG CTG GTG CAG TCT GG-3'
HuVH6A-BACK	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'	HuVH6A-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG CAG TCA GG-3'
		VH forward	
		HuJH1/2-FOR	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
		HuJH3-FOR	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
		HuJH4/5-FOR	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
		HuJH6-FOR	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'

Table I. (continued)

A. Primary amplification		B. Secondary amplification	
Vκ back		Vκ back	
HuVκ1B-BACK	5'-GAC ATC CAG WTG ACC CAG TCT CC-3'	HuVκ1B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAC ATC CAG WTG ACC CAG TCT CC-3'
HuVκ2-BACK	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'	HuVκ2-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAT GTT GTG ATG ACT CAG TCT CC-3'
HuVκ3B-BACK	5'-GAA ATT GTG WTG ACR CAG TCT CC-3'	HuVκ3B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAA ATT GTG WTG ACR CAG TCT CC-3'
HuVκ4B-BACK	5'-GAT ATT GTG ATG ACC CAC ACT CC-3'	HuVκ4B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAT ATT GTG ATG ACC CAC ACT CC-3'
HuVκ5-BACK	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'	HuVκ5-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAA ACG ACA CTC ACG CAG TCT CC-3'
HuVκ6-BACK	5'-GAA ATT GTG CTG ACT CAG TCT CC-3'	HuVκ6-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAA ATT GTG CTG ACT CAG TCT CC-3'
Vλ back		Vλ back	
HuVλ1A-BACK	5'-CAG TCT GTG CTG ACT CAG CCA CC-3'	HuVλ1A-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG TCT GTG CTG ACT CAG CCA CC-3'
HuVλ1B-BACK	5'-CAG TCT GTG YTG ACG CAG CCG CC-3'	HuVλ1B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG TCT GTG YTG ACG CAG CCG CC-3'
HuVλ1C-BACK	5'-CAG TCT GTC GTG ACG CAG CCG CC-3'	HuVλ1C-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG TCT GTC GTG ACG CAG CCG CC-3'
HuVλ2-BACK	5'-CAR TCT GCC CTG ACT CAG CCT-3'	HuVλ2-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAR TCT GCC CTG ACT CAG CCT-3'
HuVλ3A-BACK	5'-TCC TAT GWG CTG ACT CAG CCA CC-3'	HuVλ3A-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT TCC TAT GWG CTG ACT CAG CCA CC-3'
HuVλ3B-BACK	5'-TCT TCT GAG CTG ACT CAG GAC CC-3'	HuVλ3B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT TCT TCT GAG CTG ACT CAG GAC CC-3'
HuVλ4-BACK	5'-CAC GTT ATA CTG ACT CAA CCG CC-3'	HuVλ4-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAC GTT ATA CTG ACT CAA CCG CC-3'
HuVλ5-BACK	5'-CAG GCT GTG CTG ACT CAG CCG TC-3'	HuVλ5-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG GCT GTG CTG ACT CAG CCG TC-3'
HuVλ6-BACK	5'-AAT TTT ATG CTG ACT CAG CCC CA-3'	HuVλ6-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT AAT TTT ATG CTG ACT CAG CCC CA-3'
HuVλ7/8-BACK	5'-CAG RCT GTG GTG ACY CAG GAG CC-3'	HuVλ7/8-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG RCT GTG GTG ACY CAG GAG CC-3'
HuVλ9-BACK	5'-CWG CCT GTG CTG ACT CAG CCM CC-3'	HuVλ9-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CWG CCT GTG CTG ACT CAG CCM CC-3' TCT CC-3'

PCR was performed in a volume of 50 μ l using AmpliTaq polymerase (Cetus) and 500 pM of each primer for 28 cycles (1 min at 94°C, 1 min at 55°C and 2 min at 72°C), 9 separate IgM derived VH-amplifications were generated with 2 μ l random primed cDNA (equivalent to 6 μ g PBL RNA or to 7 μ g spleen RNA) as template for each reaction. For the light chain families, 6 different V κ C κ -products and 11 V λ C λ -products (C λ 2- and C λ 7-primers combined in each reaction) were obtained. All products were purified from agarose gel with the QIAex-II extraction kit (Qiagen). As input for reamplification to introduce restriction sites, 100-200 ng purified DNA-fragment was used as template in a 100 μ l reaction volume. The large amount of input, ensuring the maintenance of variability, was checked by analysis of 4 μ l of the "unamplified" PCR-mixture on agarose gel.

3.3 Construction of the primary and secondary repertoires

For the construction of the primary heavy chain and the two primary light chain repertoires, the PCR-products, appended with restriction sites, were gel purified prior to digestion and the different VH-, V κ - and V λ -families combined into three groups. The V κ C κ - and V λ C λ -fragments were digested with *Apa*LI and *Asc*I, and cloned into the phagemid vector pCES1 (Fig. 1). The VH-fragments, 1.5 μ g in total, were digested with *Sfi*II and *Bst*EII and ligated in a 100-200 μ l reaction mixture with 9 U T4-DNA ligase at room temperature to 4 μ g, gel-purified vector pUC119-CES1 (similar to vector pCES1, but with the pIII gene deleted). The desalted ligation mixture for light or heavy chain pools was used for electroporation of the *E. coli* strain TG1, to create the one-chain libraries.

The Fab library was obtained by cloning of VH fragments, digested from plasmid-DNA prepared from the heavy chain repertoires, into the plasmid collection containing the light chain repertoires. Plasmid DNA isolated from at least 3×10^9 bacteria of the VH library was digested with *Sfi*II and *Bst*EII for cloning in the vector that already contained κ and λ light chain libraries. To retain clones with internal *Bst*EII site in the V λ (this site is relatively frequent in some λ germline V-segments (22), and also in the constant domain of one of the λ families), the cloning of VHCH1 in the λ light chain repertoire containing vector was also carried out using *Sfi*II and *Nor*I cloning sites, to create a less restriction-biased V λ library.

3.4 Selection of the library

The rescue of phagemid particles with helper phage M13-KO7 was performed according to (18) on 10-L scale, using representative numbers of bacteria from the library for inoculation, to ensure the presence of at least 10 bacteria from each clone in the start inoculum. For selections, 10^{13} cfu's (colony forming units) were used with antigens immobilised in immunotubes (Maxisorp tubes, Nunc) (18) or with soluble biotinylated antigens (23). The amount of the immobilised antigens tetanus toxoid and the hapten phenyl-oxazolone (conjugated to BSA in a ratio of 17 to 1) was reduced 10-fold during subsequent selection rounds, starting at 100 μ g/ml at

round 1. Capture with biotinylated antigen in solution was used for a 100-mer peptide encoding five copies of the tandem repeat of MUC1 (24), or with human Chorionic Gonadotropin (hCG), human Luteinizing Hormone (hLH), human Follicle Stimulating Hormone (hFSH) and its chimeric derivative (hFSH-CTP, containing the carboxy terminal peptide from the hCG β -subunit fused to the β -subunit of hFSH). Antigens were biotinylated at a ratio of ten to twenty molecules NHS-Biotin (Pierce) per molecule antigen according to the suppliers recommendations. Unless stated otherwise, the antigens were used for selection at concentrations of 100 nM, 30 nM and 10 nM during round 1, 2 and 3 respectively. For hFSH-CTP 50, 15 and 10 nM was used respectively; for MUC1 peptide, 500, 100, 20 and 5 nM was used.

3.5 Screening and sequencing of clones

Soluble Fab was produced from individual clones as described before (18). Culture supernatants were tested in ELISA with directly coated antigen or indirectly captured biotinylated antigen via immobilised biotinylated BSA-streptavidin. Tetanus toxoid and pHx-BSA were coated at 10 $\mu\text{g/ml}$ in 0.1 M NaHCO_3 pH 9.6 for 16 h at 4°C. For coating of hCG and hFSH-CTP a concentration of 4 $\mu\text{g/ml}$ in 50 mM NaHCO_3 pH 9.6 was used. For capture of biotinylated antigens, biotinylated BSA was coated at 2 $\mu\text{g/ml}$ in PBS during 1 h at 37°C. After 3 washes with PBS-0.1% (v/v) Tween 20 (PBST), plates were incubated during 1 h with streptavidin (10 $\mu\text{g/ml}$ in PBS / 0.5% gelatin) (24). Following washing as above, biotinylated antigen was added for an overnight incubation at 4°C at a concentration of 0.5 $\mu\text{g/ml}$ for MUC1 peptide, 3 $\mu\text{g/ml}$ for hLH, and 0.6 $\mu\text{g/ml}$ for hFSH (binding to hCG was tested with directly coated antigen). The plates were blocked during 30 min at room temperature with 2% (w/v) semi-skimmed milk powder (Marvel) in PBS. The culture supernatant was diluted 1 or 5-fold in 2% (w/v) Marvel / PBS and incubated 2 h; bound Fab was detected with anti-myc antibody 9E10 (5 $\mu\text{g/ml}$) recognising the myc-peptide tag at the carboxyterminus of the heavy Fd chain, and rabbit anti-mouse-HRP conjugate (DAKO) (18). Following the last incubation, staining was performed with tetramethylbenzidine (TMB) and H_2O_2 as substrate and stopped by adding half a volume of 2 N H_2SO_4 ; the optical density was measured at 450 nm. Clones giving a positive signal in ELISA (over 2x the background), were analysed by *Bst*NI-fingerprinting of the PCR-products obtained by amplification with the oligonucleotide primers M13-reverse and geneIII-forward (18).

Large-scale induction of soluble Fab fragments from individual clones was performed on 50 ml scale in 2xTY containing 100 $\mu\text{g/ml}$ ampicillin and 2% glucose. After growth at 37°C to an OD_{600} of 0.9, the cells were pelleted (10 min at 2.934xg) and resuspended in 2xTY with ampicillin and 1 mM IPTG. Bacteria were harvested after 3.5 h growing at 30°C by centrifugation (as before); periplasmic fractions were prepared by resuspending the cell pellet in 1 ml ice cold PBS. After 2 to 16 h rotating head-over-head at 4°C, the spheroplasts were removed by two centrifugation steps: after spinning during 10 min at 3,400xg, the supernatant

was clarified by an additional centrifugation step during 10 min at 13,000xg in an eppendorf centrifuge. The periplasmic fraction obtained was directly used for determination of fine specificities by surface plasmon resonance or for western blot studies (described below).

For sequencing, plasmid DNA was prepared from 50 ml cultures grown at 30°C in LB-medium, containing 100 µg/ml ampicillin and 2% glucose, using the QIAGEN midi-kit (Qiagen). Sequencing was performed with the thermocycling kit (Amersham) with CY5-labeled primers CH1FOR (5'-GTC CTT GAC CAG GCA GCC CAG GGC-3') and M13REV (5'-CAG GAA ACA GCT ATG AC-3'); samples were run on the ALF-Express (Pharmacia). V-gene sequences were aligned to V-base (Tomlinson *et. al.*, V-BASE, MRC Centre for Protein Engineering, 1997, <http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html>) or to the Sanger Centre database (Sanger Centre Germline Query, 1997, <http://www.sanger.ac.uk/Data Search/gq-search.html>).

3.6 Determination of fine specificities of the anti-hormone Fab's by western blot and surface plasmon resonance

An hCG-preparation purified from urine and immuno-affinity purified recombinant hLH, hFSH and hFSH-CTP produced in CHO-cells (25,26) were used for western blot studies as was described (27). Between 0.5 and 1 µg of each hormone was loaded per lane; proteins were diluted in non-reducing sample buffer and boiled during 5 min or directly applied on gel without heat-treatment; proteins were transferred to blotting membrane by electrotransfer. Blots were subsequently incubated for 16 h at room temperature with a 10-fold diluted periplasmic fraction in PBS / 4% Marvel. Bound Fab was detected with anti-myc antibody 9E10 (5 µg/ml) and 4,000-fold diluted anti-mouse alkaline phosphatase-conjugate (Promega), using the substrates 5-bromo-1-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Boehringer Mannheim) for visualisation.

The specificity of the Fab's was further characterised by surface plasmon resonance (BIAcore 2000, Biacore). Recombinant hLH, hFSH and the urinary hCG were immobilised on the flow-cells of a CM-chip using the NHS/EDC-kit (Pharmacia), yielding a surface of 1906 RU for hLH, 1529 RU for hFSH and 1375 RU for hCG. Periplasmic fractions were diluted three-fold in Hepes Buffered Saline (HBS; 10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.05% (v/v) surfactant P20, pH 7.4) and analysed using a flow rate of 10 µl/min.

3.7 Purification of soluble Fab fragments

Fab's were obtained by refolding of the total bacterial proteins from a 50 ml culture (28). Briefly, the pelleted cells from a 50 ml induced bacterial culture were resuspended in 8 ml 8 M urea (in PBS). After sonication, the mixture was rotated head over head for 30 min and insoluble material was removed by centrifugation for 30 min at 13,000xg. The supernatant was dialysed against PBS with four buffer changes. Insoluble proteins were removed by centrifugation and the

flow through fraction, obtained by filtration through a 0.2 μm membrane, was immediately loaded on an hCG column (bed volume 0.3 ml). The column material was prepared by coupling 8.4 mg protein to one gram Tresyl sepharose according to the suppliers instructions (Pierce). The column (1 ml column material) was washed with 10 volumes 100 mM Tris, 500 mM NaCl pH 7.5, subsequently with 10 volumes 100 mM Tris / 500 mM NaCl pH 9.5 and with 2 volumes 0.9% NaCl, bound Fab was eluted with two volumes 0.1 M TEA and immediately neutralised with 0.5 volume 1M Tris pH 7.5. The Fab fraction was dialysed against PBS using a Microcon 30 spin dialysis filter (Amicon). Finally, a gel-filtration analysis was carried out on a Superdex 75HR column (Pharmacia). The yield was determined by measuring the optical density at 280 nm (using a molar extinction coefficient of 13 for Fab's).

3.8 Determination of on- and off-rate using surface plasmon resonance with crude Fab preparations

The kinetics of binding were analysed by surface plasmon resonance on three different hCG surfaces (303 RU, 615 RU and 767 RU immobilised, with 4955 RU BSA on a separate flow cell as a negative control). Fab present in crude periplasmic extracts was quantified on a high density surface of purified anti-human-Fab polyclonal antibody (Pierce) as described (29). Anti-hCG Fab's controls were purified by affinity chromatography on hCG columns as described above and used to calibrate the system.

4. Results

4.1 Design of the naïve phage antibody library

We considered a number of variables to address in the construction of a novel, very large antibody phage library: (i) the primer design was optimised for amplification of variable gene pools to maintain maximum diversity; (ii) a highly efficient two-step cloning method was developed to obtain a very large naïve library; (iii) an antibody format and compatible cloning vector were chosen, which should permit the rapid down-stream analysis of selected clones.

In order to achieve access to as many different human heavy and light chain V-region gene segments as possible, a new set of oligonucleotide primers was developed (Table I), the design of which was based on the most recent sequence information provided by the V-base (see Materials and Methods). The primers should allow efficient amplification of all commonly used V-gene segments. Further, to obtain large sized libraries (over 10^{10} in diversity), we used a two-step cloning procedure: heavy and light chain variable genes were first separately cloned as digested PCR products, and were then combined by restriction fragment cloning to form a large library of Fab fragments. This cloning procedure should be a more efficient route for library

construction than the relatively inefficient direct cloning of digested PCR-products, while avoiding the DNA instability often associated with *in vivo* recombination systems (30).

As choice of antibody format, we preferred the Fab above the scFv format, because this would allow us to develop rapid high through-put affinity-screening assays for crude antibody preparations. Many scFv's indeed form higher molecular weight species including dimers (31,32) and trimers (33), which complicate both selection and characterisation. We chose the Fab display format in which the heavy chain is linked to the phage coat protein pIII, and also carries a tag for detection and purification (see below). The light chain is expressed as separate fragment secreted into the periplasm, where it can pair with the heavy chain (34).

Fig. 1. Schematic representation (A) and polylinker region (B) of the phagemid vector pCES1.

A.

B.

To incorporate all these improvements, a new phagemid vector, pCES1 (Fig. 1), was constructed, that allows the stepwise cloning of antibody fragments in Fab format. In this vector system, the variable heavy chain region genes are cloned as VH-gene fragments; the vector

supplies all Fab's with a human gamma-1 CH1 gene. The Fd fragment is fused to two tags for purification and detection (a histidine tail for Immobilised Metal Affinity Chromatography (35) and a *c-myc*-derived tag (36)), followed by an amber stop codon (34) and the minor coat protein III of filamentous phage fd. The antibody light chain is cloned as full VLCL fragment, for directed secretion and assembly with the VHCH1 on the phage particle.

4.2 Library construction

The Fab library was constructed in two-steps. In the first step, variable region gene pools were amplified from approx. 4×10^8 B-cells from the PBLs of four healthy donors, and, as a source of possibly more heavily mutated IgM antibodies, from a segment of a (tumor-free) spleen removed from a patient with gastric carcinoma, containing approximately 1.5×10^8 B-cells (37). Only IgM-derived VH segments were amplified by using an amplification with an oligonucleotide primer located in the first constant domain of this isotype. These products were cloned into phagemid vector pCES1 for VL, and in pUC119-CES1 for VH (cloning was more efficiently in the smaller sized vector, in which gene III was deleted). The PBL and spleen derived VH, V κ and V λ -libraries were cloned separately to maintain diversity, to yield one-chain libraries in size typical for libraries made by cloning of PCR-fragments (18): 1.75×10^8 individual clones for the heavy chain, 9.4×10^7 clones for V κ , and 5.2×10^7 clones for V λ . An overview is given in Table II.

Table II. *Size and composition of the phage antibody libraries*

	Source	Library	% Insert	Size ^a
	B-cells			(cfu)
one-chain libraries	PBL	VH	64	1.8×10^8
		V κ	100	5.7×10^7
		V λ	96	4.5×10^7
	spleen	VH	75	8.0×10^7
		V κ	100	3.0×10^7
		V λ	80	1.0×10^7
Fab-library	PBL		90	1.95×10^{10}
	spleen		80	2.35×10^{10}
total size			86	4.3×10^{10}

^a Not corrected for clones without Fab encoding insert.

In the second step, the heavy chain fragments were digested from plasmid DNA isolated from the primary VH repertoire, and cloned into the vector containing the light chain repertoires (again separately for PBL and spleen derived repertoire; Table II). The libraries were combined using this efficient cloning procedure, to create a naïve Fab repertoire with 3.7×10^{10} individual clones (4.3×10^{10} recombinant clones, 86% of which have a full-length Fab insert), with 70% of clones harbouring a kappa light chain, 30% a lambda chain. All of 20 clones with full length Fab insert tested scored positive in dot-blot analysis with the 9E10 antibody to indicating an expression level of soluble Fab of at least 0.2 mg/L.

4.3 Quality control of the library by selection with a panel of antigens

We evaluated the library by selection with different antigens, the screening data of which are summarised in Table III. First, the results from three model antigens, the protein tetanus toxoid, the hapten 2-phenyloxazol-5-one (phOx) (38) and the peptide MUC1, are discussed. Three rounds of biopanning on tetanus toxoid yielded a diverse set of ELISA positive Fab's: in a series of 47 tetanus toxoid binding Fab's, at least 21 were different with regard to BstNI-fingerprint. Similarly, an extensive panel of phOx-specific Fab's was retrieved after three rounds of panning: at least 24 different clones were identified in a series of 50 ELISA positive clones. Solution capture with biotinylated MUC1 peptide resulted in the selection of 14 different antibody fragments out of 37 ELISA-positive clones selected after 3 rounds (Table III).

Table III. Overview of results of selections on a diverse set of antigens

Antigen	# ELISA positives			# different Ag-spec. clones
	round 2	round 3	round 4	
TT ^a	13/36	69/80	-	>21
phOx ^b	29/37	70/80	-	>24
MUC1 ^c	1/37	32/87	25/87	14
hCG ^d	-	24/48	34/48	8
hLH ^e	-	40/45	30/45	>21
hFSH ^f	-	30/45	40/45	6
hFSH-CTP ^g	-	16/48	17/48	7

^a TT: tetanus toxoid; ^b phOx: 2-phenyloxazol-5-one; ^c MUC1: Mucin-1 derived peptide; ^d hCG: human Chorionic Gonadotropin; ^e hLH: human Luteinizing Hormone; ^f hFSH: human Follicle Stimulating Hormone; ^g hFSH-CTP: chimeric product of β -CTP of hCG fused to β -FSH.

4.4 Rapid dissociation rate determination

With such large panels of antibodies isolated, it is crucial to have methods available to readily determine the kinetic parameters of each individual antibody-antigen interaction. Such an

assay should be robust and ideally employ non-purified antibody fragments. We tested if it would be feasible to use periplasmic fractions prepared from small scale cultures for a rapid and accurate determination of the off-rate of the antibodies using surface plasmon resonance. An example of an overlay plot with the sensorgrams from a series of tetanus toxoid specific Fab's is shown in Fig. 2. The plot of $\ln(R_0/R)$ versus time (Fig. 2, lower graphs) reveals a linear relation with slope k_d (off-rate), thereby confirming a monophasic dissociation, which can be expected for a truly monomeric Fab-fragment binding to a low density antigen surface. Using this off-rate screening assay, we determined the off-rates for the best tetanus toxoid and MUC1 specific Fab's to be in the order of 10^{-2} to 10^{-4} s^{-1} (Fig. 2).

Figure 2. Off-rate screening in BIAcore of selected Tetanus toxoid and MUC1 binding Fab fragments. Periplasmic fractions from 4 anti-tetanus toxoid clones (A) and from anti-MUC1 clones (B) were injected at $t = 115 \text{ s}$ on flow cells with immobilised tetanus toxoid and MUC1 peptide, respectively. At $t = 175 \text{ s}$ the dissociation phase started by washing with HBS-buffer. Below the sensorgram, the derivative $\ln(R_0/R)$ of two representative clones was plotted against the time (R_0 taken from $t = 175 \text{ s}$), which should be linear for a truly monophasic dissociation. Resulting dissociation rates are shown below each series of sensorgrams.

4.5 Selection of Fab antibodies against related glycoprotein hormones

As a more stringent test panel of antigens to assay the performance of the library, we chose to derive antibodies to three structurally related glycoproteins: human Chorionic Gonadotropin (hCG), human Luteinizing Hormone (hLH) and human Follicle Stimulating Hormone (hFSH) (reviewed in (39)). These hormones are heterodimers sharing an identical α -chain with 92 amino acid residues, but have β -subunits of different composition and length. The β -chain of hCG contains 145 amino acid residues, and the one from hLH only 121 residues, the latter showing 85% homology to β -hCG. The β -chain of hFSH is only 111 amino acids and shares 36% of the residues with hCG. Antibodies that specifically detect hCG have been used extensively in pregnancy tests (39) and for cancer diagnosis (40,41). A large set of antibodies to these targets would extend the limited number of hormone specific antibodies (especially against hLH), obtained using the hybridoma technology (39). The human origin of the antibodies might be beneficial when using these for imaging or therapy of testicular and bladder cancer (40,41).

Selections were thus performed on biotinylated urinary hCG, recombinant hLH, hFSH and hFSH-CTP (the latter is a chimeric molecule containing the carboxy terminal peptide of β -hCG fused to the β -chain of FSH (42)). The highest degree of enrichment in respect to the increase in the number of eluted phage particles in round 3 versus round 1 was found for hCG (10,000-fold), followed by hFSH-CTP (1,000-fold), hFSH (300-fold) and hLH (150-fold). Polyclonal phage of selected populations were tested for binding using sensorchips containing immobilised hormones (43). Polyclonal phage selected with hCG showed binding after rounds two and three of selection to all three proteins, i.e. hCG, hLH, and hFSH, with the strongest signal visible for hCG (Fig. 3A). Similar analysis of the polyclonal phage populations selected for three rounds on hFSH showed a dominance of hFSH-specific binding, while selections on hFSH-CTP yielded binders to both hFSH and hCG (Fig. 3B). Selections on hLH yielded antibodies reactive with hFSH and hCG (thus most likely anti- α -chain antibodies; marked LH(1) in Fig. 3B). When hLH was used at lower concentrations (at 10 nM in round 1 and 3 nM during the subsequent selection rounds), a signal was seen with streptavidin only (marked LH(2) in Fig. 3B), due to the selection of streptavidin-specific antibodies. Thus, this polyclonal phage screening provides a rapid test to check the overall quality of the clones in the selected repertoire, and may also be used to guide the choice of the conditions for the next selection round (43).

Figure 3. (Next page) Monitoring of selections with polyclonal phage using surface plasmon resonance. Polyclonal phage populations from round 1, 2 and 3 (R1, R2 and R3, respectively) of the selection with hCG, were analysed on flow cells with hCG, hLH and hFSH; at $t = 70$ s, $t = 400$ s and $t = 690$ s phage was injected, ending at $t = 120$ s, $t = 450$ s and $t = 740$ s respectively (A). Analysis of phage from round 3 selected with the antigens hCG, hFSH-CTP, hFSH, and hLH (the latter was selected with 100 nM hormone (coded LH(1)) or 10 nM (coded LH(2)) at round 1), using flow-cells with hCG and streptavidin (upper sensorgram) or with hFSH-CTP, and hCG (lower sensorgram); injection was started at $t = 60$ s, $t = 270$ s, $t = 500$ s, $t = 720$ s, and $t = 950$ s, and terminated at $t = 120$ s, $t = 330$ s, $t = 560$ s, $t = 780$ s, and $t = 1010$ s respectively (B).

A **B**

4.6 Specificity analysis of the selected monoclonal Fab's

ELISA of monoclonal phage antibodies revealed that three rounds of selection with hCG indeed resulted in the isolation of a high percentage (74%) of clones positive for the gonadotropin. 27% of these clones were hLH cross-reactive; none were reactive against streptavidin. *Bst*NI-fingerprint analysis of the ELISA-positive clones revealed a high degree of diversity (8 different patterns). From a representative hCG-specific (coded CG#4F) and hLH cross-reactive (CG#5C) clone, the specificity was tested in BIAcore using unpurified soluble Fab fragments (Fig. 4). Clone CG#4F gave a high response on hCG, with no visible binding to either hLH or hFSH-CTP. In contrast, clone CG#5C bound to hCG and hLH, but not to hFSH-CTP. Western blots, with the different hormones in non-reduced form, showed the specific recognition of the β -subunit of hCG by clone CG#4F, while the cross-reactive clone CG#5C reacted with the β -subunit of both hCG and hLH (Fig. 5).

Selection with the hormone hLH resulted in the isolation of hLH-specific and hCG cross-reactive clones. Examination of individual clones from selection round three in ELISA revealed a large fraction of hLH specific clones (69%), and a minor group of cross-reactive clones (16%); no streptavidin reactive clones were selected. Within the group of specific clones, a large array of different species (>21) could be discriminated with fingerprint analysis; however, all cross-reactive species had a single pattern. The unique hLH specificity was confirmed for representative clones LH#2H and LH#3G, shown in surface plasmon resonance (shown for clone LH#3G in Fig. 4); and on western blot (illustrated for clone LH#3G in Fig. 5). This Fab only recognises the intact α/β -heterodimer of hLH. Two representative clones of a pan-reactive antibody in ELISA, coded LH#1C and LH#3F, reacted in BIAcore with hFSH-CTP, hCG and hLH (shown for clone LH#3F in Fig. 4), and in western blot analysis with the α -chains from all three hormones (not shown).

When hFSH was used as antigen during selection, 6 different antibodies were isolated from the library, with one type, represented by clone FS#8B, dominating the selected population. This Fab only recognised hFSH in BIAcore (Fig. 4), and, as western blot analysis demonstrated, in particular its β -unit (Fig. 5). Further, the specificity of an α -chain binding clone, SC#2B, was confirmed in BIAcore (Fig. 4) and western blot (Fig. 5).

Upon selection with FSH-CTP 7 different α -chain specific Fab's were identified by fingerprint analysis, from which the clones coded SC#2B, SC#2F, SC#2G and SC#4G were examined in more detail. Immunoblot analysis with the recombinant Fab as detecting antibody confirmed the α -chain specificity (blot incubated with clone SC#2B is shown in Fig. 5).

4.7 Phage selected antigen-specific clones are intact Fab fragments

There have been some reports on the isolation from scFv or Fab libraries of antigen-specific single-domain or other artificial antibody fragments (44,45). Therefore, we tested the integrity of

Figure 4. Analysis of specificity of selected anti-glycoprotein hormone Fab's using surface plasmon resonance. Periplasmic fractions from clones CG#4F, CG#5C, LH#3G, LH#3F, FS#8B and SC#2B were injected at $t = 120$ s on flow-cells with immobilised hFSH-CTP, hLH and hCG, and on an empty surface; at $t = 180$ s the injection phase was stopped and the cells were washed with HBS buffer.

the selected Fab's. First, the nature of the Fab fragments in periplasmic fractions was determined in western blot. When incubated in non-reducing sample buffer, two products were detected with the 9E10 antibody, which recognises the *myc*-tag at the end of the CH1 domain (Fig. 6A): the major product is the intact Fab-molecule, in which an intermolecular disulphide bridge

Figure 5. Specificities of the Fab's determined with western blot analysis. The glycoprotein hormones hCG, hFSH and hLH were diluted in non-reducing sample buffer, and boiled (T+) or not heat-treated (T-), loaded on a 15 % SDS-PAGE, blotted onto nitrocellulose filter, and detected with the indicated Fab's. The molecular forms of the hormones recognised by the Fab's of clones CG#4F, CG#5C, LH#3G, FS#8B, and SC#2B, are indicated with arrows.

Figure 6. Examination of the integrity of produced Fab fragments on western blot. The periplasmic fractions from the indicated glycoprotein specific Fab clones were boiled in non-reducing sample buffer and loaded on 12.5% SDS-PAGE. The blots were incubated with the anti-myc mAB 9E10 (A), anti-human lambda polyclonal antibodies (B), or with anti-human kappa polyclonal antibodies (C). Affinity purified Fab was used as a control on the blot incubated with anti-myc mAB (indicated with pur Fab).

covalently links heavy and light chain fragments; the low molecular product is most likely derived from non disulphide bridge linked heavy chains. Analysis with anti-light chain sera reveals a similar pattern and shows that the clones use a nearly equal percentage of kappa and

Figure 7. SDS-PAGE of affinity purified Fab's. hCG binding Fab's produced by clones LH#1C, LH#3F, CG#5C, SC#2B and SC#2F were purified by affinity chromatography on hCG-sepharose, and analysed on a Coomassie stained 12.5% SDS-PAGE gel under reducing conditions, and for Fab SC#2B also under non-reducing conditions. As positive control, a Fab fragment made by proteolytic digestion of a human monoclonal antibody, was included (marked with +).

lambda chains (found in six and seven clones respectively of a total of 13 tested) (Fig. 6B,C). From the densities of the blots it would be unlikely that all of the light chain would be complexed as Fab. Instead, it appears that in many cases more light than heavy chain fragment is produced, which may be expected from the design of the bicistronic expression cassette. Upon purification of functional antigen-binding fragments using denaturation and refolding, followed by affinity chromatography, this excess of clones in Fig. 7). Upon reduction, equal amounts of heavy and light chain are seen, while under non-reducing conditions (shown for one clone only), the main product is represented by the disulphide linked Fab-molecule, with an equal amount of the (most likely) non-covalently linked VHCH1 and VLCL products visible. Production yields of selected hormone specific Fab's varied between 160 µg and 1.43 mg Fab per litre culture, which was in the same range as was found for the unselected Fab's (data not shown).

4.8 Use of diverse germline sequences

A panel of 14 antigen-specific Fab's was fully sequenced (Table IV; 3 anti-MUC1 antibodies positive in BIAcore on 100-mer peptide; 11 anti-gonadotropin antibodies). The heavy chain genes are derived from the four largest VH families (VH1, VH3, VH4 and VH6); the VL

Table IV. V-gene segments and CDR3-sequences used by the selected Fab's

clone	specificity	VH family	VH segment	CDR3	AA changes from germline*	VL family	VL segment	CDR3	AA changes from germline*
MUC#4	MUC1	VH4	4-39	AAAGMVD	7	V κ 2	A17	MQATHWPPIT	1
MUC#9	MUC1	VH4	4-b/4-39	PSIAAAGQVY	5	V κ 3	L6	QQYYSWPLT	10
MUC#32	MUC1	VH3	3-30	VGSGGWLYFDR	2	V κ 3	L6	QQRSHWPLT	2
CG#4F	β -CG	VH3	3-30.3	EGTATPGGTDY	2	V κ 1	L5	QQSYSTPL	7
CG#5C	β -CG/LH	VH4	4-04	GAAASYFFDY	0	V λ 6	6a	QSSHSTAVV	13
LH#1C	α	VH1	1-08	GERSNFDY	8	V κ 3	L6	QHRRT	8
LH#2H	$\alpha\beta$ -LH	VH3	3-15	DPGTIIYYYYGMDV	0	V κ 2	A19	MQALQAPLT	4
LH#3F	α	VH3	3-30.5	LYGDYVSAPDI	2	V λ 7	7b	LLYYGGARGV	6
LH#3G	$\alpha\beta$ -LH	VH3	3-15	RIAAYYYYYGMDV	0	V κ 2	A19	MQALQTPRT	0
FS#8B	β -FSH	VH6	6-01	GEHGYTSS	8	V κ 2	A19	MQALQTPPT	1
SC#2B	α	VH1	1-02	GKVGASFDY	3	V λ 2	2a2	SSYTNSTGLV	9
SC#2F	α	VH1	1-02	ASGYFPNDAFDI	6	V λ 1	1c	ESLDDSLDGVV	20
SC#2G	α	VH1	1-e	GEASYGAYNWFDI	5	V λ 2	2e	CSYAGSNTWV	9
SC#4G	α	VH1	1-24	GGYSGLA	2	V κ 2	A19	MQTLQPPWT	2

*Amino acid differences in V-gene segment, excluding the FR-1 region encoded by the primers used for cloning, and CDR3.

genes belong to one of four V κ -families or one of three V λ -families. Chain promiscuity is seen for the α -chain specific clone SC#4G, the $\alpha\beta$ -LH specific clones LH#2H and LH#3G, and β -FSH specific clone FS#8B, which all used a highly homologous V κ 2 light chain gene segment (A19, previously coded DPK15) combined with different heavy chain fragments. This promiscuity for A19 was previously found in antibodies derived from a synthetic Fab repertoire (30). The 3 anti-MUC1 antibodies use heavy and light chain genes derived from 2 different VH and V κ families; clone MUC#9 uses a VH with a cross-over of 2 segments. It is remarkable that both MUC#4 and MUC#9 VH genes use the same reading frame of the same D-segment (D6-13; with a stretch of 13 or 16 bp from this segment, respectively (46)), encoding an alanine-glycine stretch (AAAG; Table IV). This may reflect a similar mode of binding to MUC1, despite the use of a different light chain by these two clones.

4.9 Measurement of affinities with purified anti-hCG Fab's

The affinities and off-rates of affinity purified hCG reactive Fab's LH#1C, SC#2B, LH#3F and CG#5C were determined. The off-rates for most Fab's were in the order of 10^{-2} and 10^{-3} s $^{-1}$ (Table V). The off-rate values obtained using crude periplasmic fractions were in good agreement with the values found for the purified Fab's, validating the utility of the off-rate screen with unpurified Fab fragments. The affinities, 23 nM and 38 nM for the α -subunit specific antibody LH#1C and the β -subunit hCG/hLH-cross reactive antibody CG#5C respectively, are

comparable to the affinity of antibodies selected from a murine immune phage antibody library (H.d.H., B. Kazemier, et al.. unpublished); the top affinity, 2.7 nM for the α -chain specific Fab SC#2B (Table V), approaches the values of the best anti-hCG monoclonal antibodies (H.d.H., B. Kazemier, et al.. unpublished).

Table V. Affinities of anti-hormone Fab's for hCG

Clone	Preparation	k_{off} (s^{-1})	k_{on} ($M^{-1}.s^{-1}$)	K_d (M)
LH#1C	purified periplasmic fraction	$(1.04 \pm 0.04) \times 10^{-2}$ $(1.12 \pm 0.08) \times 10^{-2}$	$(4.50 \pm 0.37) \times 10^5$ nd	$(2.30 \pm 0.23) \times 10^{-8}$
SC#2B	purified periplasmic fraction	$(2.89 \pm 0.41) \times 10^{-3}$ $(2.90 \pm 0.57) \times 10^{-3}$	$(1.06 \pm 0.08) \times 10^6$ nd	$(2.71 \pm 0.25) \times 10^{-9}$
CG#5C	purified periplasmic fraction	$(1.46 \pm 0.27) \times 10^{-2}$ $(1.21 \pm 0.16) \times 10^{-2}$	$(3.76 \pm 0.71) \times 10^5$ nd	$(3.88 \pm 0.03) \times 10^{-8}$ nd
LH#3F	periplasmic fraction	$(5.84 \pm 0.08) \times 10^{-3}$	nd	nd
CG#4F	periplasmic fraction	$(2.22 \pm 0.28) \times 10^{-3}$	nd	nd
SC#2F	periplasmic fraction	2.25×10^{-3}	nd	nd

nd, not determined.

5. Discussion

This report describes the construction of a phage display library from the *in vivo* rearranged V-gene repertoire of human donors and its evaluation by selection with a panel of hapten and protein antigens. The source of antibody producing B-cells was two-fold: peripheral blood lymphocytes, which are mainly IgM-positive cells, and B cells from a human spleen. We employed an efficient two-step cloning procedure with DNA-fragments digested from plasmid DNA instead of PCR fragments, to obtain the largest naïve human Fab repertoire reported to date, with a theoretical diversity of 37 billion different clones.

The choice of the Fab format was based on the possibility to develop rapid affinity/kinetic screens. Most large libraries made to date use the single chain format for display on phage (7,20). One report described the use of a human naïve Fab library on phage (not permitting immediate screening of selected soluble Fab fragments) (30). scFv's have the tendency to form dimers and higher order multimers in a clone-dependent and relatively unpredictable way (31,32,47). As a consequence, the affinity assay used (such as BIAcore analysis) often necessitates purification of the selected antibody fragments. For example, ranking for off-rates using BIAcore is not easily possible with unpurified scFv fragments; the monomeric fraction of

selected scFv clones first needs to be purified by affinity chromatography and gel-filtration (20,48). Our data suggest that the off-rate screening of individual Fab clones using non-purified bacterial preparations yield data similar to the off-rates determined with the purified Fab fragments. Therefore, provided sufficient Fab fragment is produced, the true monomeric appearance of Fab's allows a rapid initial screen for off-rate. In combination with a concentration determination assay (which could also be carried out on BIAcore (29)), this should allow the rapid affinity determination of large series of antigen-specific Fab's. The Fab format is therefore more amenable than scFv fragments to high throughput affinity screening, and should be the preferred format when rapid affinity measurement is crucial (e.g. during affinity maturation studies).

As was postulated and observed by Griffiths and colleagues (30), the size of the antibody library dictates the probability of the selection of high affinity antibodies to the antigen. Comparison of the first naïve scFv repertoire containing 2.9×10^7 clones (18), with a recently constructed scFv repertoire of approximately 10^{10} clones (7,20), confirms this postulation: increasing the library size 500-fold resulted in approximately 100-fold higher affinities. This increase is caused by lowering the off-rates from 10^{-1} - 10^{-2} s^{-1} for fragments selected from the smaller sized library to 10^{-3} - 10^{-4} s^{-1} for those from the larger library. This is in the same order of magnitude as we observe for the off-rates of our selected antibody fragments. Since Fab fragments lack the tendency to dimerise, Fab libraries could possibly display a lower fraction of avid phage than equivalent scFv libraries library. This does not appear to have reduced the number nor average affinity of selected antibodies. An indication that antibodies from this library behave similarly or better with regards to affinity comes from a comparison of selections of two different libraries on the same two antigens under identical conditions. Antibodies to MUC1 selected from a large naïve scFv library (24) have faster off-rates than the equivalent Fab's isolated from the library described in this study. Further, they show a very distinct V-gene usage and have a different fine specificity (P.H., H.R.H. *et al.*, unpublished). Similarly, when comparing the off-rates of phage antibodies against the pancarcinoma marker Epithelial Glycoprotein-2, one of the Fab's selected from the present library appears to have a 10-fold slower off-rate than the best scFv (7) (R.C.R., H.R.H. *et al.*, in preparation).

The affinities of the selected antibody fragments is, however, very much dependent on the antigen used for selection. Sheets and colleagues reported an affinity varying between 26 and 71 nM for the selected scFv fragments specific for the anti-Clostridia botulinum neurotoxin type A fragments, whereas for antibodies to the extracellular domain of human ErbB-2, K_d 's between 0.22 and 4.03 nM were found (20). The affinities of the gonadotropin specific Fab's selected from our library varied between 2.7 and 38 nM, which is comparable to the protein binding scFv's from the naïve library made by Vaughan *et al.* (7) and Sheets *et al.* (20), and approaches the values of the best antibodies in their kind (H.d.H, Kazemier *et al.*, unpublished).

The size of the library is not only important for affinity, but it also determines the success rate of selection of antibodies against a large set of different antigens. In this respect the library performs very well: over 24 antibodies to the hapten phOx, and on average 13 antibodies against the other antigens were selected. Furthermore, the specificities of the antibodies obtained by selections on the gonadotropins are unique: due to the high degree of homology between hLH and hCG it has been very difficult to isolate hCG specific monoclonal antibodies with the hybridoma technology, whereas there are very few hLH specific antibodies (27,39). Using a straight forward selection procedure, taking no precaution to avoid the selection of cross-reactive Fab's, we have readily isolated fragments with all possible specificities: Fab's specific for any of the three hormones hCG, hLH and hFSH, and cross-reactive Fab's recognizing the common α -chain or epitopes on the β -chain shared by hCG and hLH. These selections demonstrated that antibodies directed against different epitopes within single antigen molecules can be retrieved from the library.

In the limited set of 14 clones that were sequenced, we identify antibodies with variable region genes from all large V-gene families, including VH1/3/4, V κ 1/3, and V λ 1/2, but also less frequently used segments of family VH6, V κ 2/7 and V λ 7 were retrieved. Most likely the use of an extended set of variable region gene primers, designed on the most recent sequence information of the germline V-regions, and/or the separate PCRs, combined with partially separate cloning, ensured access to a highly diverse sample of the human V-gene repertoire.

The average amino acid mutation frequency of the selected human V-genes was calculated to be the 3.95% for the VH segments (50 amino acid mutations in 1267) and 7.30% for the VL (92 out of 1260). This mutation frequency is reversed to what was reported for IgG+ B-cells (49) (10% for VH and 6% for VL). The higher mutation rate of the VL-genes in the combinatorial repertoire may serve to compensate the rather restricted natural light chain diversity. As the overall natural light chain diversity is only a fraction of the heavy chain diversity, the use of spleen versus PBL-derived lights chains, may help to provide additional light-chain based combining site diversity.

With respect to the VH segments of the selected antibodies, the mutation frequency is remarkable lower (mean 3.57($\pm$ 2.90) mutated residues per VH) than what was found in the selected scFv fragments by Vaughan and colleagues (7) (mean 7.53($\pm$ 4.25) mutated residues). The light chain segments seem to have similar mutation frequencies (Vaughan *et al.*: 8.23 $\pm$ 5.20 residues, versus 6.57 $\pm$ 5.56 residues in the here reported Fab's). Probably the IgM-specific primer, used by us for PCR, resulted in the isolation of more "naïve" VH segments.

This new phage library will be a valuable source of antibodies to essentially any target. To date we have been able to select specific antibodies to over 20 antigens tested. The antibodies may be used as research reagents or as starting point for the development of therapeutic antibodies. As the list of sequenced genomes and disease-related gene products is expanding rapidly, there will be a growing need for an in vitro and eventually automated method for

antibody isolation. As antibodies have been and will be ideal probes for investigating the nature, localisation and purification of novel gene products, this library is envisaged to play an important role in target validation and target discovery in the area of functional genomics.

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SUMMARY; SAMENVATTING

1 Exploring Antibody Engineering Technology for diagnostic applications; summary

As was stated in the Introduction, the principal aim of the thesis was to determine the value of phage display and engineering technology in the generation and modification of antibodies for diagnostic purposes. The evaluation was performed in the field of endocrinology, for diagnosis of pregnancy, and in the area of virology for the detection of the AIDS-causing Human Immunodeficiency Virus type 1 (HIV-1). The immunoassays dedicated to the detection of hormones and viruses have to be specific and sensitive, characteristics which are determined by the used antibodies. Therefore, the library derived antibody fragments were analysed on these features and compared with monoclonal antibodies made via traditional methods.

1.1 Library derived anti-hCG scFv-s

The display of antibodies on the filamentous bacteriophage M13 was set up in our laboratory with hybridoma produced monoclonal antibodies reactive against the hormone human Chorionic Gonadotropin (hCG). The idea is obvious: the combination of one type of heavy chain variable region with its partner light chain variable region within a single chain antibody, should yield antigen reactive phage, when the recombinant molecule was expressed on its surface. However, we encountered problems when using several anti-hCG and anti-HIV-1 antibodies, in that we never found antigen binding phage and derived antibody fragments.

By application of mutant analysis, as described in Chapter 1, it was concluded that a single mutation within the Frame work region 1 of the heavy chain variable region (V_H), introduced by the oligonucleotide primer used for amplification of the V regions, was responsible for this failure. Three studied anti-hCG antibodies with a subgroup IIA V_H , completely lost their capacity to bind antigen, when at position 6 the wild type residue glutamine (Q) was substituted by glutamic acid (E), a residue found frequently in murine antibodies also. The inactive mutant scFv-s turned out to be more sensitive for trypsin digestion than the wild type derivatives, which suggests incorrect folding of the mutant fragments.

Grafting of three residues located within the first two β -strands of the V_H -domain, that distinctively occur in antibodies with a of subgroup IA V_H and thereby having glutamic acid as number 6 residue, onto the "defect" subgroup IIA sequence partially restored activity of the Q6E containing scFv. Possibly the involved residue may be part of a folding nucleus, and considering the evolutionary conservation of either glutamine or glutamic acid in the two murine antibody families, this folding nucleus might even be family specific.

A similar effect on antigen binding, caused by a mutation in the Frame work region 1 of V_K , was discussed in Chapter 2. When the wild type residue methionine at position 4 was substituted by serine in two anti-hCG antibodies having a subgroup II kappa light chain, antigen-binding

could not be detected in ELISA. However, the same replacement within an anti-hCG antibody with a subgroup IV kappa light chain, and thereby having leucine as wild type residue, did not affect the binding characteristics. In contrast with the above described V_H -mutation, a low affinity binding could be detected by surface plasmon resonance on a high density surface of antigen. An intermediate trypsin resistance might be an indication for a more local distortion of the conformation rather than a completely misfolded antibody domain, which was observed with the previously described V_H -mutation. The local effect might influence the canonical structure of the nearby located LI, and resulting from this, the binding affinity.

During both studies it was important to quantify the amount of actively binding scFv in a crude periplasmic fraction. In Chapter 3, a method was developed where the concentration of antigen binding scFv was measured with surface plasmon resonance on BIAcore under mass transfer limitation conditions. When scFv was injected on a high density antigen surface, a linear relationship was obtained between the binding rate and the concentration of the antibody fragment. After construction of a single calibration curve with purified scFv, the amount of antigen binding antibody fragment in a sample could be determined rapidly by its binding rate, acquired from injection on the same surface.

For diagnosis of pregnancy, elevated levels of the glycoprotein hormone human Chorionic Gonadotropin (hCG) in urine are measured in immunoassays. Complicating factors are the presence of the closely related human Luteinizing Hormone (hLH) and of free subunits or fragments derived from the β -chain of hCG. Therefore a combination of monoclonal antibodies has to be used, by which only intact hCG will be detected. Especially cross-reactivity against hLH forms a severe problem, since both hCG and hLH use an identical α -chain, and their β -chains are 85% homologous in respect to the first 114 amino acid residues. In this context we applied phage display technology for the selection of hCG specific scFv-s. Chapter 4 describes the use of libraries, generated from the spleens of two immunised mice, for the selection of different anti-hCG scFv-s. Analysis with surface plasmon resonance and Western blot using the recombinant antibodies as detection probes, we identified a group of anti- β -core fragment, hCG-specific antibodies, as well as an antibody only recognising the bioactive molecular form of hCG, being the intact heterodimer. In spite of its cross-reactivity against hLH, its use in an assay in combination with the hCG-specific antibody fragment would detect the bioactive molecular form of the hormone specifically.

ScFv-s with other specificities were also selected, such as anti- α -subunit specific antibodies, and a group of anti- β -core, hLH cross reactive fragments. Comparison of the library derived scFv-s with those of monoclonal antibodies, which are included in a commercially available pregnancy assay, showed that both groups have similar affinities. Therefore, the same sensitivity can be expected for an assay, which will contain the recombinant antibody fragments.

1.2 Anti-HIV-1 scFv-s

The presence of Human Immunodeficiency Virus type 1 (HIV-1) in human sera is diagnosed most frequently on the major capsid protein p24. The structural protein is abundantly present in the viral genome containing capsid. Screening with p24-antigen assays enables an early identification of sera from infected individuals: with currently used immunoassays, the presence of p24 antigen may be assessed one to two weeks earlier than an antibody response against the virus. Testing on p24 is important also during treatment of patients with anti-viral drugs. The spontaneous generation of drug resistant variants leads to a reactivation of virus. In order to detect the virus in an early stage of the reactivation phase, but also during the primary infection, the applied monoclonal antibodies must have high affinities, since this feature is directly correlated with the sensitivity of the assay. Because of the heterogeneity of the different HIV-strains, the antibodies also have to recognise conserved epitopes within p24.

The selection of a number of anti-p24 scFv-s from an immune repertoire is presented in Chapter 5. After immunisation of a mouse with HIV-1 viral lysate, RNA was extracted from its spleen and used for the construction of the repertoire. One of the selected antibodies recognised an immunodominant epitope of p24, as could be concluded from pepscan analysis. A previously described hybridoma produced monoclonal antibody, with the same specificity, used the identical combination of V_H- and V_κ-regions. Therefore this scFv is one of the rare examples where the original pairing in a library derived antibody fragment was retrieved. The affinities of the selected fragments were comparable to those of monoclonal antibodies, that are currently applied in a commercial p24 antigen assay.

Instead of immunising mice, a human repertoire was made for the selection of anti-HIV-1 envelope antibodies. Chapter 6 discusses the generation of the scFv-library from just a small number of peripheral blood lymphocytes, donated by a seropositive individual. An important argument for selection of human antibodies is the difference in antigen presentation during immunisation of a mouse, when compared with virus replication in man. Also, false positive ELISA signals, observed with sporadically occurring sera containing human anti-mouse antibodies (HAMA), may be avoided by the application of human antibodies in ELISA for capturing and/or detection.

Biopanning with the envelope precursor gp160 resulted in the selection of two families of scFv-s. One group recognised a conformational epitope within the mature envelope protein gp120, the other family was specific for the transmembrane glycoprotein gp41; both structural proteins are cleaved by proteolysis from gp160. Due to a sequence homology with previously described library derived Fab-s, it was concluded that our scFv-s react with the cluster III epitope of gp41.

Competition ELISA showed that especially the epitope of the α -gp41 antibodies was recognised by a broad panel of patient sera from North American and African seropositives. Due

to the conserved nature of its epitope, the anti-gp41 scFv seems to be the most promising candidate for application in *in vitro* diagnostics.

1.3 Construction of and selections from a naïve human repertoire

The rather time-consuming immunisation and the repeated construction of immune repertoires, needed for the selection of the anti-hCG- and anti-HIV-1 -antibodies discussed in the previous Chapters, can be completely superseded by the application of a naïve repertoire. It has been reported, that depending on the library size, high affinity antibodies against any antigen can be retrieved from such repertoires.

Chapter 7 presents the construction of a huge naïve Fab repertoire, containing 37 billion phage clones. The quality was analysed by selection with two protein antigens, i.e., tetanus toxoid and the tumor associated antigen mucine-1, and the hapten pHx, and by the following affinity analysis of the selected Fab-s. As measure for the affinity, the off-rates were determined with surface plasmon resonance by injection of crude periplasmic fractions, which followed monophasic kinetics due to the purely monomeric appearance of the Fab-s.

The real challenge was formed by selection of the naïve repertoire with the glycoproteins hormones, a theme, which is running through this thesis like a continuous thread (Chapter 1 to 4). Upon selection with human Chorionic Gonadotropin (hCG) a group of Fab's was found, recognising the holo-form of this hormone specifically, as well as β -unit specific Fab's, which were cross reactive against human Luteinizing Hormone (hLH). With hLH as target molecule for selection, we retrieved Fab's, recognising the heterodimer form of the target hormone only, and a group of antibodies reactive against the α -subunit, and therefore cross-reactive against the other members of the family of glycoprotein hormones. Application of human Follicle Stimulating Hormone (hFSH) resulted in the isolation of β -subunit, hormone specific antibodies and α -unit hLH/hCG cross reactive Fab's. From all hCG-reactive antibody fragments, purified with antigen columns, we determined the kinetics of binding to antigen with surface plasmon resonance. The affinity varied between 2.7 and 38 nM, comparable with antibodies obtained from an immune repertoire or produced by hybridoma cell lines.

2 Samenvatting: evaluatie van Antibody Engineering Technologie voor toepassing in diagnostika

Zoals in de Inleiding reeds werd vermeld, was het belangrijkste doel van dit promotie onderzoek het belang van faag display en genetische manipulatie vast te stellen voor het maken en de modifikatie van antilichamen, die diagnostisch toegepast worden. De evaluatie vond plaats op het gebied van de endocrinologie, voor het vaststellen van zwangerschap, en op het gebied van de virologie, voor de detektie van het Human Immunodeficiency Virus type 1 (HIV-1), welke de ziekte AIDS veroorzaakt. De immunologische testen, die gebruikt worden voor de detektie van hormonen en virussen, moeten specifiek en gevoelig zijn, karakteristieken die bepaald worden door de in de test gebruikte antilichamen. Daarom werden de antilichaam fragmenten, die geïsoleerd werden uit de faag banken, geanalyseerd op deze beide eigenschappen en vergeleken met monoklonale antilichamen, die met traditionele methoden gemaakt zijn.

2.1 Anti-hCG scFv-s geïsoleerd uit een faag bank

De display van antilichamen op de filamenteuze bacteriofaag M13 is binnen onze groep opgezet met door hybridoma's geproduceerde antilichamen, die tegen het hormoon human Chorionic Gonadotropin (hCG) gericht zijn. Het achterliggende idee ligt voor de hand: de combinatie van een type zware keten variabele regio met de bijbehorende lichte keten variabele regio in één enkelketenig antilichaam fragment, ofwel "single chain Fv" (scFv), moet een antigeen specifieke faag opleveren, wanneer het rekombinante molecuul op diens oppervlak tot expressie wordt gebracht. Wij hadden echter veel problemen bij het omzetten van een aantal verschillende anti-hCG en anti-HIV-1 monoklonale antilichamen naar in *E. coli* geproduceerde antilichaam fragmenten: we vonden nooit antigeen bindende fagen en hiervan afgeleide antilichaam fragmenten.

Door de analyse van mutanten, welke beschreven is in Hoofdstuk 1, kon worden aangetoond, dat één enkele mutatie binnen Frame work regio 1 van de zware keten variabele regio (V_H), geïntroduceerd door een oligonucleotide primer, die gebruikt was voor de amplificatie van de V-regio's, verantwoordelijk was voor deze mislukking. Drie bestudeerde anti-hCG antilichamen met een groep IIA V_H verloren in het geheel het vermogen om antigeen te binden, wanneer op positie 6 het wild type residu glutamine (Q) vervangen was door glutamaat (E), een residu dat ook veelvuldig voorkomt in muis antilichamen. De inaktieve mutant scFv-s bleken gevoeliger te zijn voor digestie met trypsine dan hun wild type afgeleiden, wat een verkeerde opvouwing van de mutant fragmenten suggereert.

"Transplantatie" van drie residuën uit de eerste twee β -strands van het V_H -domein, die uitsluitend voorkomen in antilichamen met een groep IA V_H , en die derhalve glutamaat als positie 6 residu hebben, naar de "defekte" groep IIA sequentie herstelde de aktiviteit van de

Q6E-mutant scFv gedeeltelijk. Waarschijnlijk maakt het bestudeerde positie 6 residu deel uit van een vouwingskern ("folding nucleus"), die, gezien de evolutionaire onveranderlijkheid van ofwel glutamaat of glutamine in de twee muis antilichaam families, wellicht familie specifiek is.

In Hoofdstuk 2 is een gelijkend effect op antigeen binding beschreven, die werd veroorzaakt door een mutatie in de Frame work regio 1 van V_K . Wanneer het wild type residu methionine op positie 4 vervangen was door serine in twee anti-hCG antilichamen met een groep II kappa lichte keten, kon er geen antigeen binding in ELISA gedetekteerd worden. Dezelfde substitutie in een anti-hCG antilichaam met een groep IV kappa lichte keten, en derhalve met leucine als wild type residu, had echter geen effect op de antigeen bindings karakteristieken. In tegenstelling tot de boven beschreven V_H -mutatie, kon er hier wel een laag affiene binding met "surface plasmon resonantie" op een oppervlak met een hoge antigeendichtheid aangetoond worden. Een intermediaire trypsine resistentie kan een aanwijzing zijn voor een lokale verstoring van de structuur, in plaats van een volkomen verkeerd gevouwen antilichaam domein, zoals die werd waargenomen bij de hiervoor beschreven V_H -mutatie. Het lokale effect kan invloed hebben op de kanonieke structuur van de dichtbij gelegen LI-loop, en als gevolg hiervan, op de bindingsaffiniteit.

Gedurende de beide studies was het belangrijk om de hoeveelheid antigeen bindend scFv in periplasmatische extrakten, dat wil zeggen in ongezuiverde vorm, te bepalen. Hoofdstuk 3 beschrijft een methode, waarbij de concentratie van antigeen bindend scFv werd gemeten onder massa diffusie gelimiteerde omstandigheden met "surface plasmon resonantie" op het BIAcore systeem. Wanneer scFv geïnjecteerd werd op een oppervlak met hoge antigeen dichtheid, werd er een lineair verband verkregen tussen de bindingssnelheid en de concentratie actief bindend antilichaam fragment. Uit een eenmalig gemaakte ijklijn, verkregen met gezuiverd scFv, kon de hoeveelheid antigeen bindend antilichaam fragment in een monster snel bepaald worden aan de hand van de bijbehorende bindingssnelheid, die verkregen kan worden door injectie van dit monster op hetzelfde oppervlak.

Voor het vaststellen van zwangerschap worden verhoogde concentraties van het glycoproteïne hormoon human Chorionic Gonadotropin (hCG) in urine gemeten met immunologische testen. De aanwezigheid van het nauw verwante human Luteinizing Hormoon (hLH) en van vrije subunits of fragmenten afgeleid van de β -keten van hCG compliceren de metingen. Daarom wordt een combinatie van monoklonale antilichamen gebruikt, die alleen het intacte hCG detekteert. In het bijzonder de kruisreactiviteit met hLH vormt een groot probleem, omdat zowel hCG en hLH een identieke α -keten bezitten, terwijl hun β -ketens over de eerste 114 aminozuren 85% homoloog zijn. In dit verband pasten wij faag display toe, met het doel hCG specifieke scFv-s te selekteren. In Hoofdstuk 4 wordt het gebruik van immuun banken beschreven, die gemaakt zijn met de milten van twee geïmmuniseerde muizen, voor de selectie van verschillende anti-hCG specifieke scFv-s. Uit de analyse met "surface plasmon resonantie" en Western blot met de verschillende rekombinante antilichamen voor detektie, konden we een

groep anti- β -core fragment, hCG specifieke antilichamen identificeren, als ook een antilichaam, dat alleen de bioactieve moleculaire vorm, namelijk de intacte dimeer, van hCG herkent.

ScFv-s met andere specificiteiten werden eveneens geselecteerd, zoals anti- α -subunit specifieke antilichamen, en een groep van anti- β -core, hLH kruisreactieve fragmenten. Vergelijking van de scFv-s geïsoleerd uit de immuunbanken met monoklonale antilichamen, die hun toepassing vinden in commercieel verkrijgbare zwangerschapstesten, toonde aan dat beide type antilichamen vergelijkbare affiniteiten hebben. Derhalve kan eenzelfde gevoeligheid verwacht worden van een assay, die de rekombinante antilichaam fragmenten zal bevatten.

2.2 Anti-HIV-1 scFv-s

De aanwezigheid van het Human Immunodeficiency Virus Type-1 (HIV-1) in humane sera wordt meestal vastgesteld aan de hand van het capside eiwit p24. Het structurele eiwit is overmatig aanwezig in het capside, die het virale genoom bevat. Door screening met p24 antigeen tetsten is het mogelijk in een vroeg stadium sera van geïnfecteerde individuen te identificeren: met heden ten dage gebruikte testen kan de aanwezigheid van het p24 antigeen één tot twee weken eerder aangetoond worden dan een antilichaam respons tegen het virus. Het testen op p24 is eveneens van belang gedurende de behandeling van patiënten met anti-virale medicijnen. De spontane evolutie van medicijn-resistente varianten leidt tot een reaktivatie van het virus. Om het virus in een vroege fase van de reaktivatie fase te detecteren, maar ook gedurende de primaire infectie, moeten de in de test toegepaste monoklonale antilichamen een hoge affiniteit bezitten, omdat deze eigenschap direct gecorreleerd is met de gevoeligheid van de test. Ten gevolge van de heterogeniteit van de verschillende HIV-stammen, is het tevens van belang dat de antilichamen geconserveerde epitopen van p24 herkennen.

De selectie van een aantal anti-p24 scFv-s uit een immuun bank staat weergegeven in Hoofdstuk 5. Na immunisatie van een muis met een viraal lysaat van HIV-1-virus, werd RNA geïsoleerd uit de milt en vervolgens gebruikt voor de constructie van het repertoire. Eén van de geselecteerde antilichamen herkent een immuundominant epitoom van p24, zoals uit de pepscan analyse geconcludeerd kon worden. Een voordien beschreven hybridoma geproduceerd monoklonaal antilichaam met dezelfde specificiteit, gebruikte de identieke combinatie van V_H - en V_K -regio's. Vandaar dat deze scFv één van de zeldzame voorbeelden is, waarbij de originele paring in een uit een immuun bank geïsoleerde antilichaam fragment terug gevonden wordt. De affiniteiten van de geselecteerde fragmenten waren vergelijkbaar met die van monoklonale antilichamen, die toegepast worden in een commercieel verkrijgbare p24 antigeen test.

In plaats van het immuniseren van muizen, is er een humane immuunbank gebruikt voor de selectie van antilichaam fragmenten gericht tegen HIV-1 envelop afgeleide eiwit-antigenen. Hoofdstuk 6 behandelt de constructie van de scFv bank van slechts een klein aantal perifere bloed lymfocyten, die gedoneerd waren door een seropositief individu. Een belangrijk argument

om humane antilichamen te selekteren is het potentiële verschil in antigeen presentatie gedurende immunisatie van een muis, in vergelijking met de virus replikatie in de mens. Eveneens kunnen, door toepassing van de humane antilichaam fragmenten in ELISA voor capture en/of detectie, vals-positieve ELISA signalen vermeden worden, die het gevolg zijn van sporadisch voorkomende sera met humaan anti-muis antilichamen (HAMA).

Biopanning met het envelop precursor eiwit gp160 resulteerde in de selectie van twee families van scFv-s. Eén groep herkende een conformationele epitoom van het rijpe envelop eiwit gp120, de andere familie was specifiek gericht tegen het transmembraan glycoproteïne gp41; beide structurele eiwitten worden door proteolyse uit gp160 gevormd. Aan de hand van een sequentie homologie met eerder beschreven Fab-s, die eveneens geïsoleerd waren uit een immuun bank, kon geconcludeerd worden dat onze scFv-s reageren met de kluster III-epitoom van gp41.

Competitie ELISA toonde aan dat in het bijzonder de epitoom van de de anti-gp41 scFv-s herkend wordt door een groot panel van sera van Noord-Amerikaanse en Afrikaanse seropositieven. Ten gevolge van het sterk geconserveerde karakter van diens epitoom, lijkt de anti-gp41 scFv de meest geschikte kandidaat voor toepassing in een *in vitro* diagnostische test.

2.3 De konstruktie van en de selectie uit een naïeve humane bank

Het tamelijk tijd-verslindende immuniseren en het herhaaldelijk maken van immuun banken, die nodig waren voor het selekteren van de anti-hCG- en anti-HIV-1-antilichaam fragmenten, die beschreven zijn in de voorgaande hoofdstukken, kan in het geheel vervangen worden door toepassing van een naïef repertoire. Uit publikaties blijkt dat afhankelijk van de grootte van de bank hoog affiene antilichamen tegen ieder antigeen verkregen kunnen worden uit dergelijke banken.

Hoofdstuk 7 behandelt de konstruktie van een zeer grote naïeve Fab bank, die 37 miljard faag klonen bevat. De kwaliteit werd geanalyseerd door de selectie met twee eiwit antigenen, welke zijn tetanus toxoid en het tumor geassocieerde antigeen mucin-1, en het haptene pHx, en de daaropvolgende affiniteitsbepalingen van de geselecteerde Fab-s. Als maat voor de affiniteit werd de snelheid van dissociatie van het antigeen-antilichaam complex bepaald met "surface plasmon resonantie" door de injectie van periplasmatische frakties; de dissociaties verliepen met een monofasische kinetiek ten gevolge van het pure monovalente karakter van de Fab-s.

De echte uitdaging was de selectie van de naïeve bank met de glycoproteïne hormoon afgeleiden, het thema, wat als een rode draad door dit proefschrift (Hoofdstuk 1 tot en met 4) loopt. Selectie met human Chorionic Gonadotropin (hCG) leverde een groep Fab-s op, die de holo-vorm van het hormoon specifiek herkende, als ook β -keten specifieke Fab-s, die kruis reageerden met human Luteinizing Hormone (hLH). Bij gebruik van hLH voor selectie werden Fab-s gevonden, die alleen de dimeer van het gebruikte hormoon herkenden, evenals een groep antilichamen gericht tegen de α -keten, en derhalve kruisreactief tegen de andere leden van de

familie van glycoproteïne hormonen. Toepassing van human Follicle Stimulating Hormone (hFSH) resulteerde in de selectie van β -keten, hormoon specifieke antilichamen en eveneens van α -keten, hLH/hCG kruisreactieve Fab-s. Van alle hCG-reaktieve antilichaam fragmenten werd, na zuivering met antigeen kolom, de kinetiek van antigeen binding gemeten met "surface plasmon resonantie". De affiniteit lag tussen de 2.7 en 38 nM, wat vergelijkbaar is met die van antilichamen verkregen uit een immuun bank of die geproduceerd worden door hybridoma cellijnen.

EPILOGUE: DISCUSSION

Diagnostic value of the recombinant antibody fragments and their engineering; general conclusions

The technology of phage antibody library display was developed in the beginning of the 90s, and at present is well accepted in the academic environment. Yet, this is one of the first extensive studies to date in which phage antibodies to a series of main diagnostic targets have been isolated, and the utility of the technique is shown outside the research laboratory environment. This is caused by the combination of the experimental difficulty of the procedure and the scepticism and resistance of more traditional immunologist to this change in technology.

As was shown in the studied cases of this thesis, we can state that the **phage antibody repertoire technology** yields antibody fragments with similar specificities and affinities as found with **hybridoma technology**. This can go as far as isolating the nearly identical Variable gene sequences, as was found in a single case: a phage-library selected anti-HIV p24 phage scFv antibody fragment contained identical V_H and V_L gene sequence as those found in a previously described monoclonal antibody. But mostly antibodies of slightly different genetic make-up were identified, but with a speed and ease that compares favourably with the hybridoma technology. For example, in the case of the hCG antigen, the spectrum of fine specificity found in phage repertoire derived antibodies matched perfectly that of hybridoma-derived antibodies. For the construction of the phage repertoires, only two mice were immunised with hCG, whereas thirty two (labour intensive) fusions were needed for the generation of the different monoclonal antibodies via hybridoma technology. Besides the time advantage for the phage approach, two other major bonuses for the technology come from the possibility to prepare unlimited quantities of the selected poly- or monoclonal phage (from the stored bacterial stocks), and to vary at will the *in vitro* selection method, enabling the isolation of infrequent or rare variants. To illustrate this last point, we cite the difference that we encountered when using biopanning on immobilised p24 versus selecting with biotinylated p24. In the latter case many different antibody variants were selected versus only one single type of scFv for the panning procedure. Similarly, *in solution* selection using hCG gave the highest degree of variation in respect to the specificity, while with coated hCG antigen only β -core specific scFv's were found. The use of soluble antigen enables the selection on affinity in a controllable manner, because the applied antigen concentration functions as a threshold. Unlike hybridoma derived antibodies, selected phage antibodies come directly with their clonal genetic message. Therefore rapid identification of the DNA type or fingerprint of each individual selected phage antibody may serve as additional test or complementary assay to test the diversity of a selected population. This is illustrated by an example from the anti-hCG scFv story: at the time of screening of the selected scFv's, material of the related antigens hLH and hSFH were not available yet, but the different types of antibodies were discriminated readily by their fingerprints. Analysis of their specificity with the related hormones confirmed the classification previously made by fingerprint analysis. Thus,

when comparing with the hybridoma technology, the cloning of phage antibodies from the repertoires made from the spleen cells of immunised animals is generating antibodies more rapidly, the antibodies are easier to analyse and the spectra of fine specificities and genetic composition of the selected phage antibodies are at least as broad if not more diverse. We foresee that the use of hybridomas, for scientific, technical, ethical as well as financial reasons, will eventually be surpassed by the use of an *in vitro* selection procedure. However, nearly a decade after the initial publication on the concept of the use of *in vitro* libraries by Milstein and Winter to by-pass the animal-made antibodies, it can not be ignored that 95% of the research laboratories still use the hybridoma technology. We contribute this to the difficulties in establishing the phage technique in a non-molecular biological laboratory, and also to the time-consuming constructing of the phage antibody library every time after immunisation. The immunisation route requires the antigen to be non-toxic and immunogenic to the host, and is time consuming: immunization costs 4 to 12 weeks at least; the construction of an immune repertoire takes at least another 4 to 6 labour-intensive weeks. Most notable achievements in the immune library field have addressed the characterization of the *human* immune response in disease, using patient derived immune repertoires for the selection, as exemplified in this thesis by our human scFv-s directed against the HIV-1 envelope proteins. Due to its speed and ease over more conventional techniques to study natural humoral responses, this will be an area which will be used more extensively in the future, despite the loss of original combination of the antibody's variable regions in the cloning procedure.

The true revolution, however, must come from the use of non-immune library sources. The repeated immunization and construction of libraries may be avoided by the use of a large antibody repertoire, from which mediate to high affinity antibodies against any type of antigen are retrieved, as exemplified by the description in this thesis of the large 'naive' phage Fab library. Indeed, the selection from such a naive repertoire permitted the direct generation and identification of monoclonal antibody fragments in less than a month. Most impressive were the results obtained when the large naive Fab library was used for selection with glycoprotein hormone hCG and relatives. Antibodies recognising hCG only were obtained without application of special selection protocols: even anti-hLH specific Fab's were selected, a species which is extremely difficult to isolate using hybridoma technology. Due the use of a very large phage library (37 billion clones), the affinities of the recombinant Fabs were comparable to affinities of antibodies made by hybridoma technology or selected from an immune repertoire. All antibodies were isolated by one scientist in approximately three months, including the complete characterisation on fine specificity, affinity and sequence. For comparison, this is the minimal time needed for immunisations only. Already, several companies have initiated phage antibody selection services to customers wishing to rapidly gain access to phage antibodies, and with the increasing spread of these large naive phage libraries in the scientific community, the use of phage antibodies is bound to by-pass animal use for antibody generation in the future. With the

apparent scope of automating the *in vitro* selection procedures, we anticipate that the isolation of antibodies to defined antigens in the future will be a matter of days rather than months. It is without doubt that this will be of impact in all fields of science in which antibodies are used as probes, reagents or therapeutics.

Besides the possibilities offered by the *in vitro* antibody generation and selection procedures, the *genetic engineering* of the available antibody genes forms a challenging opportunity for the future. Both engineering for higher expression, for improved affinity and for provision of a novel effector function may be envisaged. For example, by *in vitro* mutagenesis of the antibody sequence, we identified a key residue in the certain murine V_H genes which are extremely important for folding. Knowledge of the crucial residues in protein folding and stability, will eventually be useful to increase production levels and thus reduce costs with which antibody-based products may be made. Further, it may aid in building qualitatively better phage libraries. Mutagenesis of residues localised in the CDRs or at random within the antibody variable regions, in combination with sensitive selection methods permitting discrimination between low and high affinity antibodies, may enable the generation of higher affinity antibody variants. Several examples of picomolar affinity phage antibodies have already been described in reports from the States. The *in vitro* selection procedure appears less restricted than the immune system itself by the affinity that can actively be selected for.

Phage antibodies are bare antigen-binding modules, and come without effectors. There is now, however, a very large list of proteins or peptides the antibody may be genetically fused to, to provide a purification tag, an effector or a detection tag. Aiming at the application in diagnostics, the opportunities of genetic engineering were demonstrated elegantly in our laboratory (P. Oudshoorn, B. van Lith and P. Hiemstra unpublished data) and more recently by others. By fusion of the enzyme alkaline phosphatase from *E. coli*, to the carboxyterminus of one of the anti-hCG scFv-s, an *in vivo* produced antibody conjugate was obtained, which could be used in immunoassays without further purification. As another example, genetic fusion to a peptide sequence that was *in vivo* biotinylated in *E. coli*, yielded an antibody fragment which could be captured as a functional molecule on a streptavidin coated surface. The biotinylated scFv also gained avidity and thereby affinity by binding to soluble streptavidin. Finally, fusion of the His₆-tag facilitated coating of scFv to a polystyrene surface, without losing its capacity to bind antigen. Such downstream phage antibody engineering is necessary to provide the crucial detection or effector mechanism for use of the antibody.

In conclusion it can be stated that both the application of repertoire technology and the possible engineering techniques are extremely valuable for the generation of diagnostic reagents. The technology might enhance the development of a completely new generation of diagnostic products, especially when the skills and efforts of experts in both types of disciplines, i.e., genetic engineering and assay development, will be combined.

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Curriculum vitae

Hans de Haard werd op 11 juli 1959 geboren te Beek bij Ubbergen. In 1977 behaalde hij het eindexamen HAVO, en, na één jaar HTS werktuigbouwkunde in s' Hertogenbosch gedaan te hebben, werd begonnen met de opleiding HBO-B biochemie te Oss. Na het behalen van het diploma in 1981, werd de avondstudie HTS richting chemie in 1983 met succes afgesloten.

Van 1982 tot begin 1984 was de auteur werkzaam bij de afdeling Hepatologie van de Katholieke Universiteit Nijmegen, waar hij onder leiding van Prof. dr. Sing Hiem Yap meehielp aan de promotie van dr. Hans Princen en dr. Han Moshage. In de periode 1984 tot eind 1985 werd de scholing in en de interesse voor de Moleculaire Biologie opgedaan bij de afdeling Moleculaire Biologie van het Nederlands Instituut voor Zuivelonderzoek te Ede onder de bezielende leiding van Prof. dr. Willem de Vos. Vervolgens kreeg hij een betrekking bij het diagnostika bedrijf Organon Teknika (onderdeel van AKZO-NOBEL), alwaar hij onder leiding van dr. Piet Boender binnen de unit DRL-E onderzoek verrichtte aan het hepatitis nonA-nonB-virus.

Na de verhuizing van Organon Teknika in 1989 naar Boxtel was de auteur als projectleider binnen de groep Moleculaire Biologie van de Biosciences Research Unit (BRU) verantwoordelijk voor de klonering en expressie van antigenen in *E. coli*.

In 1991 startte het promotie onderzoek, gedeeltelijk gefinancierd met een PBTS-subsidie, die door dr. Chris van Eekelen (als Unit-Manager van de BRU) en dr. Peter van Weezenbeek (Projectleider binnen de toenmalige Biotechnology Group van Organon) aangevraagd was, en dat uitgevoerd is bij Organon Teknika.

Vanaf begin 1997 werkte hij in de groep *CESAME* van de vakgroep Pathologie aan de Universiteit Maastricht.

Sinds januari 1998 is de auteur als manager werkzaam binnen de unit Functional Biomolecules van de groep Biotechnology van Unilever Research Laboratorium Vlaardingen.

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