Chapter 2

Improved anti-IgG and HSA affinity ligands: Clinical application of VHH antibody technology

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Abstract

Large scale, highly specific purification of valuable proteins from blood and removal of undesirable components promise to have wide therapeutic applications. Moreover, depletion of bulk proteins from blood is a prerequisite for clinical proteomics. Here we describe the development of specific, high affinity Camelid antibody fragments (VHH) derived from immune libraries for purification and depletion of the bulk proteins HSA and IgG from human serum and plasma for therapeutic and research purposes. The anti-IgG VHH improved depletion of IgGs from blood substantially over the classical used method based on proteinA. To demonstrate the better performance of the VHH based IgG depletions, we analyzed the presence of auto-antibodies in human plasma before and after depletion from two groups of autoimmune disease patients, notably Goodpasture (GP) and Systemic Lupus Erythmatosus (SLE). VHHs can be produced efficiently and cost effectively in *S. cerevisiae*, a GRAS organism. A good manufacturing process (GMP) for purification of these VHHs has been developed as well. Moreover, as VHHs are single protein chains, they can be coupled relatively easy to solid matrices. These three factors are important for developing affinity purification medications.

Introduction

Affinity chromatography has revolutionized the development of new techniques in pharmaceutical science and biotechnology, where it is applied as an improved method to purify valuable substances for the more common liquid chromatography separation techniques, as size-exclusion and ion-exchange chromatography (Hage, 1999). Also clinical and research laboratories have gained much interest in this separation technique. Besides methods such as direct analyte detection and removal of highly abundant proteins that obscure the analysis of less abundant proteins, affinity chromatography can also be used to purify certain highly valuable blood components or alternatively remove harmful components involved in diseases from the blood of patients. Some damaging substances, like endotoxins and drugs, are usually removed by hemodialysis although this method is often not very efficient (Kragh-Hansen et al., 2002; Otagiri, 2005). Clearly, more specific and efficient methods to remove particular damaging substances from blood for treatment of specific diseases would be advantageous. Procedures to improve the efficiency of hemodialysis in treatment of drug overdoses, or the removal of cytokines and endotoxins, such as lipopolysaccharides, are for a large extend focused on the use of the binding characteristics of human serum albumin (HSA) (Kragh-Hansen et al., 2002; Otagiri, 2005; Zimmermann et al., 1999). Therefore, pure and large amounts of HSA are needed for which affinity chromatography offers an ideal solution. Columns that have been developed for research purposes to purify or deplete HSA from blood, such as dye or antibody based columns (Gianazza and Arnaud, 1982; Pieper et al., 2003b; Steel et al., 2003; Travis and Pannell, 1973), are not suitable for this purpose because of the low specificity, or are very expensive in the case of antibody columns.

The use of proteinA based IgG affinity chromatography for the treatment of autoimmune diseases has recently been approvedby the Food and Drug Administration (FDA). Although proteinA is a well-known and frequently used protein for IgG purification, there are several unfavorable characteristics, such as high handling costs and safety aspects (Fassina et al., 2001), which makes it less suitable for clinical applications. Furthermore, it lacks specificity for all IgG subclasses, especially IgG3 (Eliasson et al., 1988). This prevents total auto-antibody depletion in some auto-immune diseases that are known for the presence of IgG3 specific auto-antibodies, such as Systemic Lupus

Erythematosus (SLE), Dilated Cardiomyopathy (DCM) and Primary Biliary Cirrhosis (PBC) (Amoura et al., 2000; Rigopoulou et al., 2005; Staudt et al., 2002). Consequently, there is a high interest in novel methods that enable the efficient, cost-effective and reliable purification of this important group of blood proteins.

Antibodies derived from animals belonging to the species of *Camelidae* are a very promising tool to be applied in this field. Besides classical antibodies, these species possess antibodies that lack the light chain (Hamers-Casterman et al., 1993). Therefore, the antigen binding domain (VHH) of these antibodies consists of only one domain, which offers several advantages over conventional antibodies, such as easy cloning to make highly diverse libraries and high production in *Escherichia coli* and *Saccharomyces cerevisiae* (Frenken et al., 2000), which makes them economically attractive. Furthermore, they represent the smallest antigen binding domains derived from antibodies (Muyldermans, 2001), and they are more stable than conventional antibodies (Dolk et al., 2005b; van der Linden et al., 1999) or their derivatives, making them extremely suitable for affinity chromatography (Verheesen et al., 2003). As these VHHs can be produced efficiently in *S. cerevisiae*, a GRAS organism, a wide range of therapeutic applications for these antibody fragments can be envisaged.

Here we describe the development of specific, high affinity VHHs against the bulk protein HSA and IgG, which can be used.for purification and depletion of the bulk proteins HSA and IgG from human serum and plasma, for therapeutic and research purposes. In a study with blood samples of GP and SLE auto-immune disease patients, the performance of the VHH column was compared to a proteinA based affinity column. In GP patients the depletion of IgG with the VHH affinity column performed at least equally well as the proteinA based column. In SLE patients, an auto-immune disease with a prevalence of IgG3 subtype specific auto-antibodies (Amoura et al., 2000), the IgG depletion with the VHH affinity column resulted in total depletion of reactive auto-antibodies whereas this was not accomplished in each individual patient sample when the ProteinA based column was used. Thus, VHH based affinity chromatography offers a technology that can be used in clinical laboratories for the development of specific and cost-effective affinity ligands for removal or purification of specific substances and can therefore greatly facilitate the progress in research and medicine. This could pave the road for improved

treatment of autoimmune disease patients and can also be used to remove microbial toxins and other harmful substances from blood, as we demonstrated recently for sepsis (ElKhattabi et al., (Submitted)).

Materials and methods

Library construction and selection of HSA and IgG specific VHHs

The anti-HSA specific VHHs were selected out of a phage display library constructed from B-lymphocytes of Llamas immunized with muscle extract using two consecutive rounds of panning with purified HSA (Sigma, Zwijndrecht, The Netherlands). The anti-IgG specific VHHs were selected out of a library constructed of Llamas immunized with total IgG. Selection was performed via panning on different purified subclasses of human IgG. Individual clones were subsequently screened in ELISA format as described before (Marks et al., 1991). Anti-IgG clones were screened for binding of all four IgG subclasses and the anti-HSA clones were screened on purified HSA (Sigma, Zwijndrecht, The Netherlands). Of each clone a DNA fingerprint was performed using the restriction enzyme Hin*F*I (van Koningsbruggen et al., 2003). The DNA of clones with a unique restriction pattern were sequenced (Sanger et al., 1977).

VHH production of selected clones

The best performing VHHs were cloned into a yeast expression vector and subsequently produced in *S. cerevisiae* as described before (Frenken et al., 2000; Thomassen et al., 2005). Secreted VHHs were purified from the fermentation medium on an ion exchange column.

Coupling of VHH to solid support

Purified VHH was coupled to a matrix using NHS (N-hydroxysuccinimide) coupling chemistry. Ligands containing primary amino groups couple directly to the active ester of NHS to form a chemically very stable amide linkage (Hermanson, 1993). As base matrix Sepharose (GE healthcare, Chalfont St. Giles, United Kingdom) was used, because it is known for its low non-specific binding. After purification, the antibody fragments were dialyzed to NHS coupling buffer (0.1 M Hepes pH 8.0). Prior to coupling of the VHHs to NHS, the matrix was washed with cold demineralized water acidified with acetic acid to pH3. Then the matrix was washed twice with NHS-coupling buffer. The washed matrix was mixed with the antibody solution and incubated overnight at 4°C head over head or 1 hour at room temperature. Subsequently, the gel material was filtered over a sintered glass filter and the non-reacted groups of the gel material were blocked with NHS block buffer (0.1M Tris pH 8.0) for 1

hour at room temperature. The coupled medium was washed using alternate low and high pH (3x10 column volumes PBS pH 2 and 3x10 column volumes PBS pH 7.4). The coupling efficiency was determined on SDS-PAGE with samples before and after coupling.

Dynamic capacity measurement

The dynamic capacity of the affinity matrices was determined on an AKTA explorer 100 (Amersham Biosciences, Roosendaal, The Netherlands). Column volume that was used for these tests was 400µl. The column was equilibrated with PBS pH 7.4 at a flow of 150 cm/hr. As sample for these experiments purified HSA and purified human IgG (Sigma, Zwijndrecht, The Netherlands) was used. Bound ligand was eluted with elution buffer (PBS pH adjusted to 2.1). The eluted fractions were immediately neutralized with one-twentieth volume 2M Tris buffer pH 8.0. The dynamic capacity was determined using peak integration of the elution peak.

BIAcore analysis

The affinities of the anti-IgG and anti-HSA VHH were determined using a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) in combination with a CM5 sensor chip (BIAcore, Uppsala, Sweden). Different concentrations of VHH (5, 25, 50, 250, and 500nM) were run over a low antigen density surface of around 1000 RU at a flow rate of 30µl/min. For the anti-HSA VHH purified HSA (Sigma, Zwijndrecht, The Netherlands), and for the anti-IgG VHH purified total IgG and purified IgG subclasses was used. Association and dissociation were measured for respectively 3 and 15 minutes. Regeneration was achieved by washing with 10mM HCl for 3 min. KD values were calculated with the BIAevaluation software.

Depletion of ligands from human plasma

This experiment was performed with the same settings as described for the dynamic capacity measurement. Column volume for these tests was 5ml. One ml Human serum (Sigma, Zwijndrecht, The Netherlands) was diluted 1:100 in PBS and run on the anti-IgG VHH column (capacity 10mg/ml). The non-bound fraction was subsequently run on the anti-HSA VHH column (capacity 12mg/ml). Bound protein was eluted with elution buffer (PBS pH2.1). To determine the efficiency of depletion, samples were evaluated on SDS-PAGE.

Furthermore, Western blots were performed using a monoclonal anti-HSA antibody (Sigma, Zwijndrecht, The Netherlands) and a polyclonal anti-human IgG antibody (Jackson Immunoresearch laboratories, West Grove, USA).
To compare the performance of the anti-IgG column to the most commonly used technique to remove and purify IgG in biotechnology and medicine, the same procedure was also performed on a proteinA column (HiTrap) (Amersham Biosciences, Roosendaal, The Netherlands). For Western blot analysis, IgG subclass specific antibodies were used (Sanquin, Amsterdam, The Netherlands).

2D-gel electrophoresis

To remove salts and lipids, samples were treated with the Clean-up kit (Amersham Biosciences, Roosendaal, The Netherlands) according to the instructions of the manufacturer. Protein pellets were dissolved in 450µl rehydration solution (7M urea; 2M thiourea; 4% CHAPS; trace bromophenol blue; 0.5% (w/v) DTT; 0.5% (v/v) ampholytes pH3-10 non linear; 1.2% (v/v) destreak). Immobiline Dry strips pH3-10 of 24cm were rehydrated overnight with the protein samples and overlaid with 2ml Coverfluid in an Immobiline Drystrip reswelling tray. The first dimension of isoelectric focusing was run on an IPGphor system (Amersham Biosciences, Roosendaal, The Netherlands). Optimal protein focusing was achieved by starting at 500V for 1 hr, followed by 1500V for another hour. Subsequently, a constant voltage of 8000V was applied until a total of 48kV. Thereafter, the strips were equilibrated in equilibration buffer (50mM Tris/HCl pH8.8; 6M urea; 30% glycerol (v/v); 2% SDS (w/v)) containing 65mM DTT for 15 minutes followed by an incubation with 135mM iodoacetamide for 15 minutes. Second dimension was performed with lab-cast 24cm 12.5% polyacrylamide gels. Strips were loaded onto the gels and sealed with a solution of 1% agarose (w/v) containing a trace of Bromophenol Blue. The gels were run over night on the Ettan DALT Twelve system (Amersham Biosciences, Roosendaal, The Netherlands) at 1W/gel till the Bromophenol Blue dye front reached the bottom of the gel.
Gels were silver stained according to the Shevchenko protocol (Shevchenko et al., 1996).

IgG depletion and analysis of autoimmune disease plasma

Plasma of GP or SLE patients was diluted 100 times in PBS or HRP sample diluent (INOVA Diagnostics, San Diego, U.S.A.), respectively. Threehundred µl diluted plasma was incubated with 60µl protA sepharose or 60µl VHH anti-IgG sepharose (capacity 10mg/ml) at 4°C for 1.5 hours. The non-bound fraction of both columns and the diluted plasma input were analyzed for the presence of auto-antibodies with ELISA. The GP plasma samples were tested on coated glomerular basement membrane (GBM) and SLE plasma samples were tested with a Quanta LiteTM Chromatin kit (INOVA Diagnostics, San Diego, U.S.A.) according to the instructions of the manufacturer. Furthermore, the SLE samples were analyzed on SDS-page and on Western blot with IgG1, IgG3 (Sanquin, Amsterdam, The Netherlands) and total IgG (Sigma, Zwijndrecht, The Netherlands) specific antibodies. The whole procedure was performed *in triplo*.

Results

Selection and characterization of anti-HSA and -IgG specific VHHs

Our approach uses VHH antibody fragments to develop improved methods to remove or purify proteins from blood for therapeutic and research applications. To prove the value of these antibody fragments, HSA and IgG specific VHH antibody fragments were selected using phage display techniques via two consecutive rounds of panning.

To obtain VHHs that recognize all four subclasses of human IgG, the selection strategy was to switch IgG subclass in consecutive selection rounds, to direct the selection to clones specific for conserved epitopes between these subclasses. Furthermore, elution of the bound clones was performed by low pH shock, as this is preferably used for elution of bound material in affinity chromatography. The output of the selections was screened for binders by ELISA, and a Hin*F*I DNA fingerprint was performed to identify unique clones. The selection output of both selections was screened in ELISA for clones that recognized their target antigen.

VHH	Antigen	ka (1/Ms)	kd (1/s)	KA (1/M)	KD (M)
Anti-IgG	IgG1	4.76E+05	1.86E-03	2.55E+08	3.92E-09
	IgG2	7.26E+05	2.41E-03	3.01E+08	3.32E-09
	IgG3	6.64E+05	4.39E-03	1.51E+08	6.61E-09
	IgG4	5.01E+05	2.85E-03	1.76E+08	5.68E-09
	IgG	7.27E+05	1.62E-03	4.40E+08	2.27E-09
Anti-HSA	HSA	4.55E+05	4.89E-03	9.29E+07	1.08E-08

Table 1: Affinity data of the anti-IgG and anti-HSA VHHs for their respective antigen obtained with the BIAcore.

For the anti-HSA VHHs, twelve different antibodies purified from 50ml *E. coli* cultures were screened on columns using diluted human serum to identify the best performing VHHs for this specific application. For the anti-IgG VHHs, twenty different clones were obtained, of which two recognized all four IgG subclasses. These two anti-IgG clones and the four best performing anti-HSA clones were cloned into a yeast expression vector and subsequent production in *S. cerevisiae* resulted in VHHs, without any tag, secreted in the growth medium. The produced VHHs were purified from the medium on an ion exchange column (Frenken et al., 2000) and subsequently immobilized on a solid support via NHS coupling chemistry. After coupling, the dynamic capacity of the affinity matrices was determined on an AKTA explorer 100 with pure

antigen. The clones that performed best in this experiment were further evaluated. The dynamic capacity of the best performing anti-HSA affinity column typically fell in the range of 8-10mg HSA. For the best performing anti-IgG column this was in the range of 12-15mg human IgG per ml affinity matrix in a settled matrix bed. The affinity of the anti-HSA and -IgG clone for their respective antigen was determined with surface plasma resonance. The anti-HSA and anti-IgG clone recognized their respective antigen with nanomolar affinity (Table 1), which is comparable to classical antibody affinities. The affinity of the anti-IgG clone was also tested separately on all four purified human IgG subclasses, which revealed that the VHH had a broad cross reactivity for all IgG subclasses. Furthermore, species specificity of the clones was also assessed. The anti-HSA clone recognized mouse serum albumin but not from rabbit and rat (data not shown). The anti-IgG VHH did not recognize total IgG from mouse, goat and rabbit (data not shown).

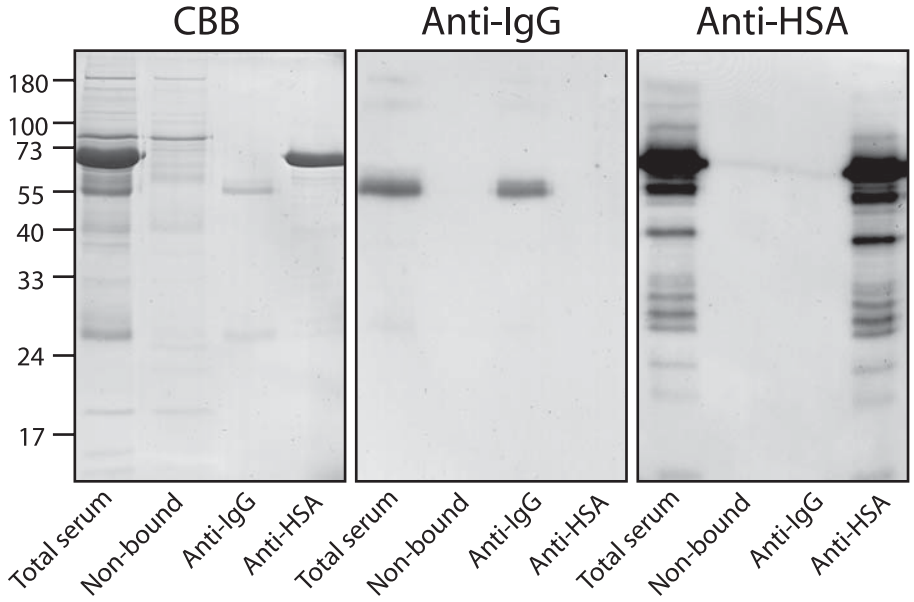


Figure 1: Assessment of the depletion of IgG and HSA from human serum. Total serum (lane 1), the non-bound fraction (lane 2) and the bound fractions of the VHH anti-IgG (lane 3) and anti-HSA (lane 4) columns were analyzed on a coomassie (CBB) stained SDS-page and on immuno blot with a commercial monoclonal antibody against IgG and HSA.

Depletion of antigens from human serum by affinity chromatography

The assessment of the obtained anti-HSA and anti-IgG clones showed a high affinity and specificity for their antigen, and functionality of the antibody fragments when immobilized onto a solid surface via primary amino groups. These matrices were used to deplete and purify HSA and IgG from human serum. First, the anti-IgG column was used to remove all IgG subclasses. Subsequently,the non-bound fraction was run over the anti-HSA column to remove HSA. Bound material was eluted by a pH shock. The whole procedure was followed on the AKTA (data not shown). This resulted in a non-bound, an IgG and a HSA fraction. These samples were analysed on SDS-page and Western blot with commercially available anti-HSA and anti-IgG specific antibodies (Figure 1). This clearly showed that no HSA or IgG was detected in the non-bound fraction. Interestingly, the anti-HSA blot of total serum showed numerous additional bands that ran primarily below the molecular weight of HSA. These proteins were also removed from the serum with our anti-HSA column. As serum contains numerous HSA fragments and modifications of HSA (Steel et al., 2003), this result indicates that this column, which contains a single monoclonal antibody, recognizes and depletes all these HSA products.

2D analysis of the obtained samples

The preceding results clearly demonstrate the depletion efficiency of our columns. To further assess the specificity of the VHH columns, the samples were further evaluated with 2D gel electrophoresis followed by MS analysis of relevant protein spots. First, the purified IgG and HSA fractions were evaluated for impurities. About 15μg of the purified IgG fraction and 50μg of the purified HSA fraction was used for this evaluation. In the IgG fraction, the IgG heavy and light chain could clearly be distinguished and no additional protein spots were observed (Figure 2). This clearly shows the specificity of the anti-IgG column. In contrast, in the HSA purified fraction several spots could be seen below and above the molecular weight of HSA (Figure 3). The most prominent protein spots were analyzed with MS and all these spots were identified as HSA. This is in agreement with the extra bands seen on Western blot with the commercial anti-HSA antibody, which are all depleted by the VHH anti-HSA column (Figure 1). The data confirm that these bands are fragments or modified forms of HSA, and furthermore illustrate the specificity and

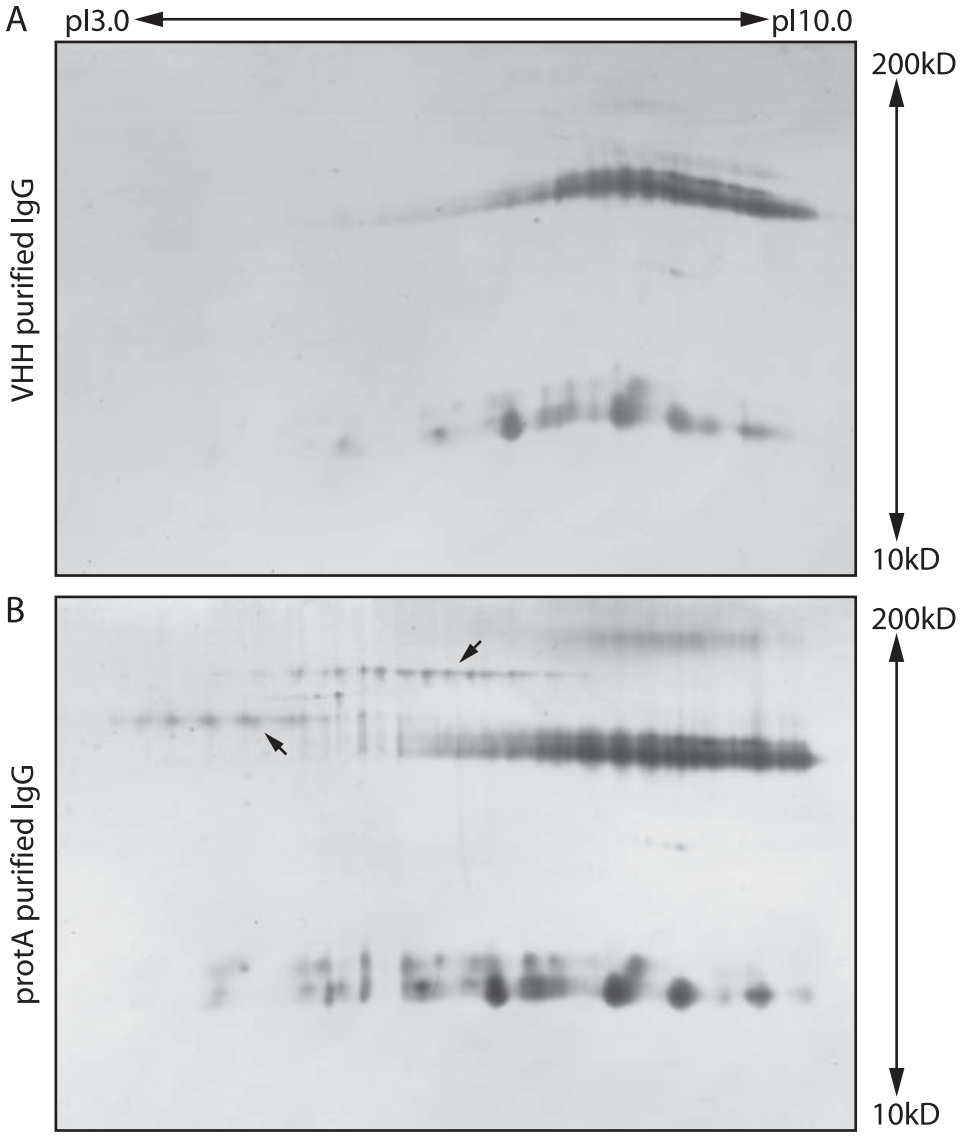


Figure 2: IgG from human serum purified with a VHH based affinity column (A) and a proteinA based column (B). Arrows indicate additional protein spots in the IgG sample purified with the proteinA affinity column.

efficiency of the VHH anti-HSA column to remove HSA, its fragments and modified products from serum.

The specific removal of abundant proteins from serum should increase the amount of spots that can be detected on a 2D gel. To evaluate this effect, the non-bound fraction was compared with the total serum sample. One hundred μg of both samples was analyzed on a 2D gel (Figure 4). These gels confirmed the data shown in figure 1. The use of the VHH affinity columns efficiently

depleted HSA and the IgG heavy chain from serum. In the depleted serum sample, a clear increase was observed in the amount of spots that could be detected. Removal of HSA and IgG revealed spots originally masked by these bulk proteins, especially in the case of HSA. Furthermore, the total amount of protein per spot increased. At the molecular weight of the light chain no total depletion was seen. This result can be explained by the presence of non-heavy chain bound light chain in blood (Abe et al., 1998; Hannam-Harris and Smith, 1981).

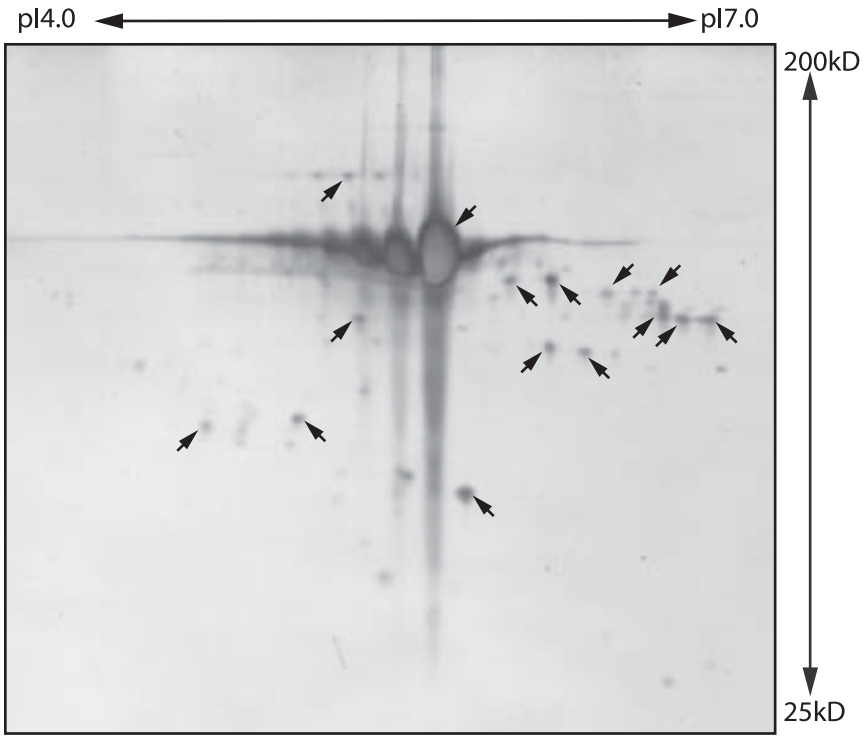


Figure 3: 2D gel of purified HSA from human serum eluted from the VHH anti-HSA column. Arrows indicate spots that were chosen, based on the relative spot abundance, for MS. All spots were positively identified as HSA.

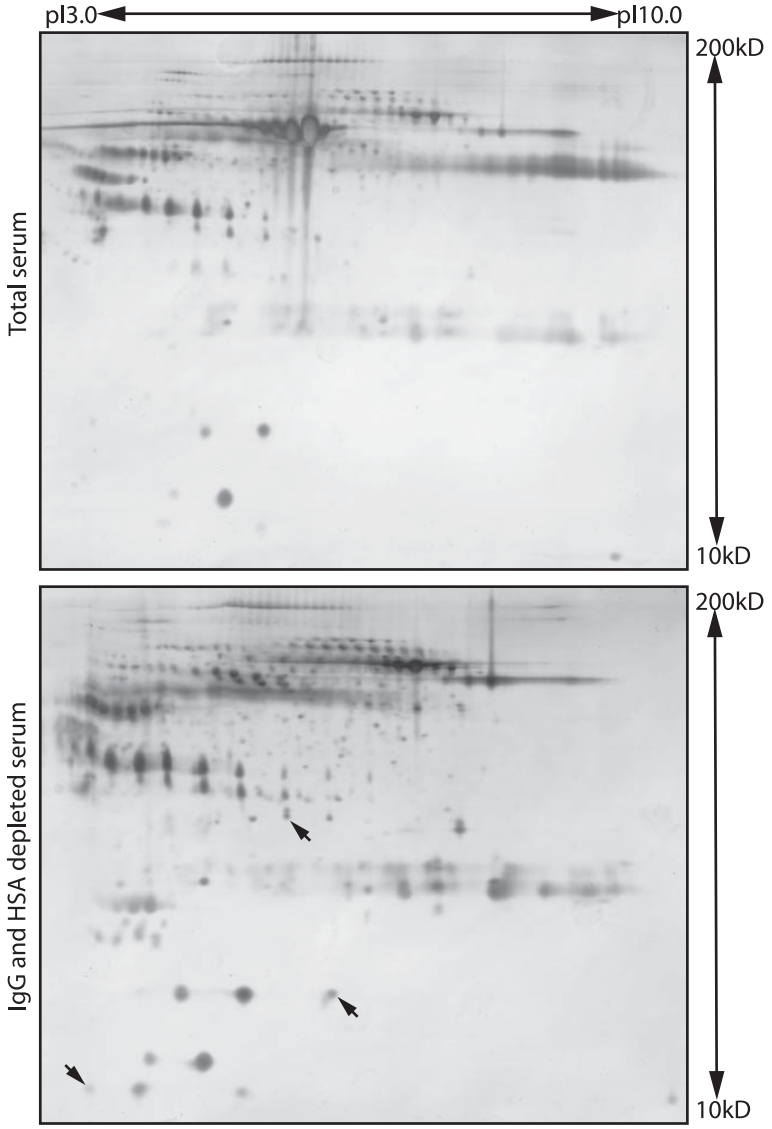


Figure 4: 2D gels of 150μg total serum and 150μg IgG and HSA depleted serum. The gels clearly show an increase in resolution and detection of proteins in the depleted human serum fraction. Arrows indicate examples of spots not seen in the total serum sample.

Performance of the anti-IgG VHH column compared to a proteinA based column

The most widely used method in affinity chromatography to isolate immunoglobulin G from biological samples is the application of proteinA from *Staphylococcus aureus* as the ligand. However, it is well known that this protein does not bind all IgG subclasses equally well (Eliasson et al., 1988). To show the potential of the VHH based anti-IgG column, its performance was compared to the proteinA method. Therefore, affinity columns of both ligands were used on the AKTA explorer 100 and non-bound and elution fractions were obtained as described before. Analysis of these samples on Western blot with total IgG and subclass specific IgG commercial antibodies (Figure 5) clearly showed that the anti-IgG VHH column efficiently depleted all IgG subclasses whereas the proteinA column removed only a small fraction of IgG3 and not all IgG4. Furthermore, analysis on 2D gel of the purified IgG fraction obtained with the proteinA column showed that this sample contained additional proteins (Figure 2B), which were not seen with the VHH column (Figure 2A).

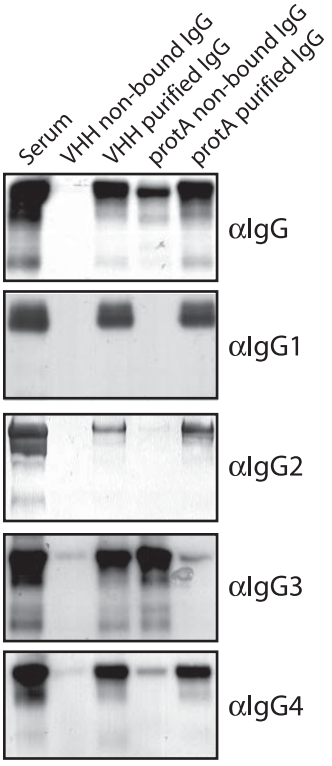


Figure 5: Increased performance of the anti-IgG VHH affinity column compared to a proteinA affinity column. The total serum sample was run on a SDS-page next to the non-bound fraction of the anti-IgG VHH and the proteinA column and the purified fractions of both columns. The depletion of IgG was evaluated on western blot with an antibody against human IgG and subclass specific antibodies. The VHH affinity column depleted all IgG subclasses from human serum, whereas the proteinA column was not able to bind IgG3 and was furthermore less efficient in depleting IgG4.

Application of the VHH anti-IgG affinity column in treatment of autoimmune diseases

Next to treatment of autoimmune diseases with plasmaphoresis and immuno suppressive therapy, specific methods to remove the self-reactive antibodies would be of great advantage. To show the possible applicability of our VHH affinity columns in therapeutic applications and to compare it with an accepted standard, GP and SLE autoimmune disease plasma were used in a set of proof of principle studies.

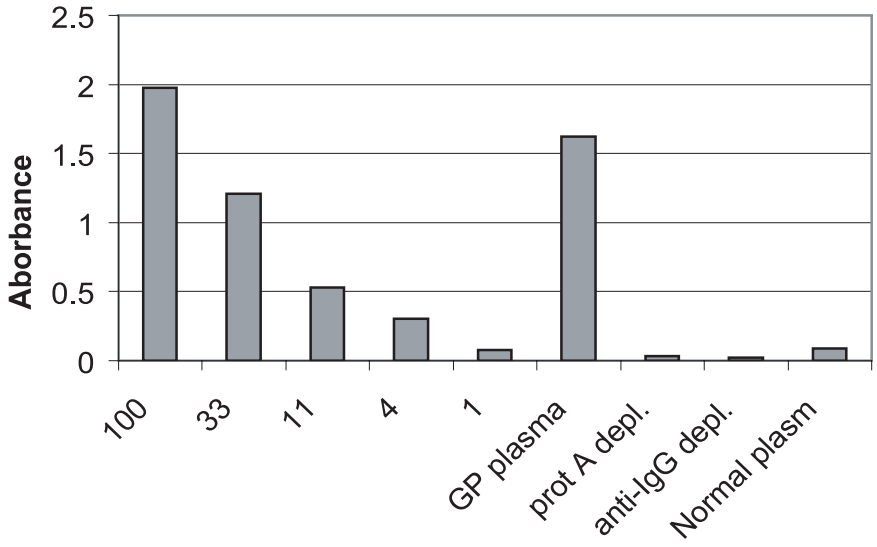
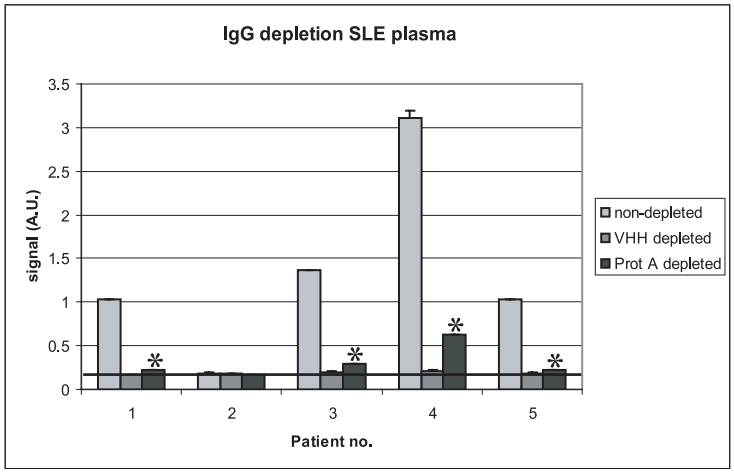


Figure 6: ELISA for measurement of auto-antibodies present in plasma of GP patients against the GBM. Depletion of IgG from plasma of Goodpasture patients with the VHH anti-IgG affinity column reduces the obtained reactivity signal to a minimum and performs at least comparable to the proteinA affinity column.

From plasma of patients suffering from these diseases IgG was removed with proteinA based or VHH based affinity ligands. The presence of self-reactive antibodies in the plasma was tested before and after depletion with dedicated ELISA kits. This showed that the VHH column performed at least equally well as the proteinA based column (Figure 6) with a sample of a GP patient. The VHH and protA column treated GP plasma showed a reduction of antibody reactivity till levels obtained with control plasma.

Remarkably, the plasma samples of SLE patients treated with the VHH based affinity ligand contained less SLE related auto-antibodies compared to the samples depleted with the proteinA affinity ligand (Figure 7), as the proteinA based depletion always gave higher signals than those obtained with the VHH based depletion.

A



B

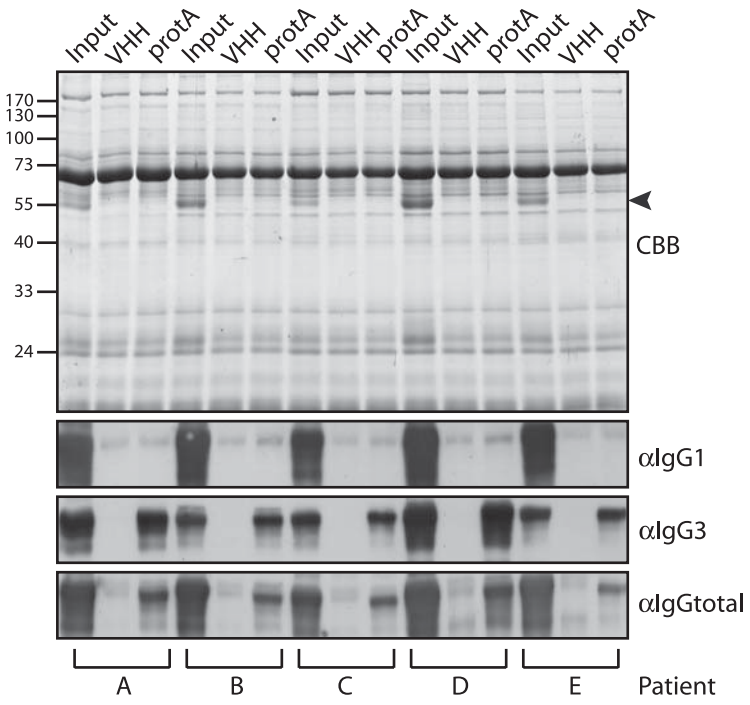


Figure 7: Representative picture of the superior IgG depletion from SLE plasma patients with the VHH anti-IgG affinity column compared to a proteinA affinity column. The ELISA for measurement of auto-antibodies against chromatin with the Quanta Lite Chromatin kit (A) shows the reduction of auto-antibodies present in plasma of SLE patients when treated with the VHH anti-IgG and proteinA affinity columns. The VHH affinity column performs better in all depicted patients with a positive ELISA signal in the non-depleted sample (p-value students-t-test ≤ 0.003) and reduces the obtained signal to background levels (black line). The depletion is analyzed on SDS-page (arrowhead) and Western blot (B) with antibodies against IgG1, IgG3 and total IgG. The increased performance of the VHH affinity column is due to the total depletion of all IgG subclasses, whereas proteinA does not deplete IgG3.

The performance of the IgG depletion was subsequently evaluated on a CBB stained gel and Western blot with IgG specific antibodies. No difference in non-specific protein loss was seen with both columns on the CBB stained gel. Furthermore, both columns depleted IgG1 with comparable efficiency, indicating that the maximal capacity of both columns was sufficient. However, again there was an enormous difference between the columns in the depletion of IgG3. Intriguingly, the SLE plasma sample that contained most IgG3 showed the highest difference between the two columns in the presence of auto-antibodies after depletion. This clearly shows the beneficial properties of the VHH based anti-IgG columns over the proteinA based affinity ligand in treatment of auto-immune diseases.

Discussion

HSA and IgG specific VHHs were selected out of two phage display libraries in two consecutive rounds of panning. The selected VHHs were tested for antigen specificity and affinity and subsequently immobilized on a solid surface and evaluated for their ability to purify and deplete their respective antigens from human serum. The performance was evaluated using 1D- and 2D-gel electrophoresis, immuno blot and BIAcore. Furthermore, the performance of the anti-IgG affinity column was compared to protein A and the potential of the anti-IgG column for medical applications was shown in a pilot experiment.

HSA and IgG specific VHHs with nanomolar affinity (Table 1) could be selected out of a phagemid immune library in a fast and directed manner. Phage display allows the selection of specific antibodies that are suitable for predetermined applications, simply by adjusting the selection and screening protocols (Dolk et al., 2005a; Verheesen et al., 2003). To obtain antibodies capable of binding all four subclasses of IgG the selection protocol was designed to drive the selection towards conserved epitopes between these subclasses. Therefore, the antigen used in sequential selection rounds was switched from one subclass to another. Furthermore, the selection outputs were screened with purified fractions of all subclasses of IgG. Before the affinity of the selected VHHs was determined, their performance was first tested in the application that they were selected for. This strategy was chosen because the affinity, although important, is not the only prerequisite for obtaining a good affinity column. For instance Chemical coupling of antibodies can cause loss of antigen binding capacity.

The obtained antibodies were very efficient in the depletion of their target antigens from human serum (Figure 1). Furthermore, the obtained antigens HSA and IgG could easily be eluted from the support. Analysis of the purified protein samples showed no proteins that were not related to the target antigen. The absence of protein contaminations in the purified antigen samples further illustrates the specificity of this method (Figure 2 and 3). Although total depletion of the IgG heavy chain was observed with the VHH anti-IgG column, a portion of proteins running at the molecular weight of the light chain remained visible. This indicates that the selected antibody binds to the heavy chain of IgG. The presence of soluble IgG light chain in human plasma can at

least in part explain this result. Furthermore, the light chains of IgA and IgM could be responsible for this observation.

The depleted serum sample resulted in 2D gels with more detectable protein spots (Figure 4). This is comparable to results obtained by other groups with conventional antibodies and proteinA (Pieper et al., 2003b; Steel et al., 2003). However, because of the relatively low production costs of the antibody fragments and their high stability, the use of VHH based affinity columns is especially attractive for clinical applications. This potential of the anti-IgG affinity column is further illustrated by the removal of self-reactive antibodies from GP plasma (Figure 6) with comparable results as obtained with a proteinA based column, and from SLE plasma with improved auto-antibody removal as obtained with proteinA (Figure 7).

Although proteinA recognizes IgG molecules within several species, this shows that the anti-IgG VHH affinity ligand has great potential to replace proteinA for large-scale purification and depletion of IgG molecules in humans. The broad cross reactivity for the different IgG subclasses (Figure 5) could enable treatment of auto-immune patients caused by any IgG subclass, and is expected to outperform proteinA based columns in treatment of diseases caused by subclass IgG3 (Amoura et al., 2000; Rigopoulou et al., 2005; Staudt et al., 2002). An indication that IgG3 removal in auto-antibody diseases might be of high importance is the association of the increase of IgG3 auto-antibodies in SLE with active nephritis (Amoura et al., 2000) and the finding that particularly IgG3 auto-antibodies in PBC causes more severe biochemical and histological disease (Rigopoulou et al., 2005). Ultimately, autoimmune patients would be helped best with a method that can specifically remove the IgGs that cause the disease. The combination of the superior characteristics of VHH antibodies in affinity chromatography, together with phage display to select antibodies that can recognize only one subtype of IgG or even anti-idiotypic antibodies would enable this. Selection of anti-idiotypic antibodies for SLE is currently in progress. By modification of the selection and screening protocol such specific affinity ligands could be obtained, not only for autoimmune diseases, but also for other diseases caused by harmful substances in the blood such as sepsis (ElKhattabi et al., (Submitted)).

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