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Recombinant Production of IgA Antibodies

Doctoral Thesis

Submitted by

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I. Acknowledgements

To my family, friends and colleagues for their love and support!

“If you love a flower that lives on a star, it is sweet to look at the sky at night.
All the stars are a-bloom with flowers...”
Antoine de Saint-Exupéry, The Little Prince

II. Abstract

Immunoglobulins of isotype A (IgA) play a key role in mucosal immunity and are promising new immunotherapeutic candidates. However, the exploration of their *in vivo* potential is often hampered by difficulties in obtaining enough material. This has two major reasons: first, the expression systems used often produce low IgA titers; second, there is still no generically applicable purification procedure available for IgA downstream processing. In the work presented in this thesis we switched the anti-HIV-1 antibodies 3D6 and 4B3 from their natural IgG1 isotype to IgA1 and established recombinant Chinese hamster ovary (CHO) cells. The generated cell lines were cultivated under serum-free conditions and stably expressed both IgA1 antibodies as polymeric IgA by combining IgA heavy chains, light chains and the joining (J) chain. Both 3D6-IgA and 4B3-IgA cell lines exhibited substantial differences in IgA productivity. To understand potential expression limitations better we broadly characterized the growth characteristics of the generated cell lines as follows: IgA productivities in long-term culture, gene copy number (GCN), mRNA levels, immunofluorescence microscopy, flow cytometry, analysis of intracellularly expressed polypeptides as well as secreted IgA product and gained novel insights. Furthermore, in search of an IgA downstream strategy, we developed a purification scheme employing an immobilized camelid anti-human alpha-chain VHH ligand and isolated both recombinant IgAs at high purity and yield in a single chromatographic step. Results were compared with a second approach in which we developed a multistep purification process consisting of an affinity step based on immobilized jacalin followed by anion exchange and hydrophobic interaction chromatography. In further experiments we aimed to generate the naturally occurring, mucosal IgA variant (secretory IgA) and successfully associated dimeric and polymeric IgA with recombinant human secretory component *in vitro*.

III. Zusammenfassung

Immunglobuline des Isotypen A (IgA) spielen eine wichtige Rolle in der Immunität der Schleimhäute und sind vielversprechende neue immuntherapeutische Kandidaten. Dennoch ist die Erforschung des *in vivo* Potentials oft erschwert durch Schwierigkeiten genug Material zu erhalten. Dies hat zwei Hauptgründe: erstens, produzieren die verwendeten Expressionssysteme niedrige IgA Titer; zweitens, gibt es noch immer keine allgemein einsetzbare Reinigungsprozedur für das Downstream Processing von IgA. In der hier präsentierten Arbeit haben wir die anti-HIV-1 Antikörper 3D6 und 4B3 von ihrem natürlichen IgG1 Isotypen zu IgA1 konvertiert und diese in rekombinanten Chinesische Ovarienzellen (CHO) exprimiert. Die generierten Zelllinien wurden unter serumfreien Bedingungen kultiviert und produzierten beide IgA1 Antikörper stabil als polymere IgAs durch Kombination der IgA schweren Ketten, leichten Ketten und des Joining (J) Peptides. Die 3D6-IgA und 4B3-IgA Zelllinien wiesen substantielle Unterschiede in ihren IgA Produktivitäten auf. Um mögliche Expressionsunterschiede besser verstehen zu können, charakterisierten wir die beiden Zelllinien anhand eines breiten Methodenspektrums wie: IgA Produktivitäten in Langzeit Kultur, Kopienzahl der rekombinanten Gene und mRNA, Immunfluoreszenzmikroskopie, Durchflusszytometrie, Analytik der intrazellulären Polypeptide sowie auch sezerniertes IgA Produkt. Darüber hinaus haben wir ein Reinigungsschema für das IgA Downstream Processing entwickelt bei dem ein immobilisierter, kamelider anti-humaner alpha-Ketten VHH Ligand eingesetzt wurde und konnten damit beide rekombinanten IgAs in einen einzigen chromatographischen Schritt mit hohem Reinheitsgrad und Ausbeute erhalten. Die erzielten Resultate wurden mit einem zweiten Ansatz verglichen in welchem wir einen Reinigungsprozess mit mehreren Zwischenschritten entwickelten. Bei diesem Ansatz wurde zunächst immobilisiertes Jacalin verwendet, dann Anionenaustauschchromatographie und zuletzt Hydrophobe Interaktionschromatographie. In weiteren Experimenten konnten wir das natürlich vorkommende, mukosale IgA (sekretoische IgA) herstellen indem wir dimeres und polymeres IgA *in vitro* mit einer rekombinant exprimierten sekretorischen Komponente assoziierten.

IV. Abbreviations

AEX	Anion exchange chromatography
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BiP	Binding protein
cDNA	Complementary DNA
CDR	Complementary determining regions
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
COPI	Coatomer protein I
COPII	Coatomer protein II
C region	Constant region
dhfr	Dihydrofolate reductase
dIgA	Dimeric IgA
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
EDEM	ER degradation-enhancing 1,2-mannosidase-like protein
eIF-2	Eukaryotic initiation factor 2,
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERQC	ER quality control
Fab	Fragment antigen binding
Fc	Fragment crystallizable
G418	Geneticin
gDNA	Genomic DNA
Glc	Glucose
GlcNAc	N-acetylglucosamine
GPI	Glycosylphosphatidylinositol
HC	Heavy chain
HCCF	Harvested cell culture fluid
HIC	Hydrophobic interaction chromatography
hSC	Human secretory component
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRES	Internal ribosome entry site
JC	Joining chain
LC	Light chain
μ	Specific growth rate
mAb	Monoclonal antibody
Man	Mannose
mRNA	Messenger RNA
MTX	Methotrexate
PBS	Phosphate buffered saline
pcd	Picogram per cell per day
PDI	Protein disulfide isomerase
PEI	Polyethyleneimine
pIgA	Polymeric IgA
pIgR	Polymeric immunoglobulin receptor
q_P	Specific productivity
qPCR	Real-time polymerase chain reaction
RB	Russell body
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SC	Secretory component
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sIgA	Secretory IgA
SRP	Signal-recognition particle
tRNA	Transfer RNA
UF/DF	Ultrafiltration/diafiltration
UPR	Unfolded protein response
V region	Variable region
VH	Light chain variable region
VL	Heavy chain variable region

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VI. Aims and rationale of the presented thesis

The human 3D6-IgG and 4B3-IgG antibodies were originally developed by hybridoma technology (Grunow et al., 1988; Buchacher et al., 1994; Kunert et al., 1998). Although both antibodies do not possess the highly effective cross-clade neutralizing properties of broadly neutralizing antibodies, both 3D6-IgG and 4B3-IgG recognize a highly conserved epitope at the gp41 Env glycoprotein of the human immunodeficiency virus type 1 (HIV-1), termed principal immunodominant domain (PID). The reason for the conservation of PID has been attributed to its essential functions in the viral life cycle, such as virus fusion with the cell membrane and entry into the target cell (Merat et al., 1999), as well as its participation in the noncovalent association between gp41 and gp120 to form the gp160 Env complex of HIV-1. It was shown that the presence of PID's loop structure is essential and Env processing was disrupted when the cysteine loop was removed (Dedera et al., 1992). Furthermore, mutations in the PID loop resulted in errors during intracellular processing of gp160 (i.e. non-cleavage of gp160 into gp41 and gp140), destabilization of the gp160 complex and gp120 shedding (Merat et al., 1999).

HIV is predominantly transmitted through mucosal surfaces of the female and male genital tracts, although 70-90% of all HIV infections are acquired by heterosexual transmission (Mestecky and Fultz, 1999). During sexual intercourse, HIV must first transverse a thick mucus layer before encountering its target cells (CD4+ T cells, dendritic cells and macrophages) and breaching the mucosa in the female genital tract (Mascola and Montefiori, 2010). At normal conditions the vaginal flora produces lactic acid to pH~4, which reduces HIV's diffusivity (1,000-fold slower than in water) through the resident human cervicovaginal mucus and therefore its infectivity. However, during sexual intercourse, semen temporarily neutralizes the vagina to pH 6-7, which increases HIV's diffusivity tremendously (to only 15-fold slower than water) (Lai et al., 2009) and therefore increases the chance of infection. In the mucosal compartment polymeric IgA is the major contributor to humoral immunity and was shown to block transcytosis of HIV-1 across the epithelial barrier (Bomsel et al., 1998). Furthermore, the polymeric structure of IgA is thought to allow virus aggregation into large complexes which were thought to limit regional virus diffusivity and spread. Such a reduced HIV flux may enhance protection by other factors in cervicovaginal mucus (e.g. defensins, lysozyme, lactoferrin, secretory leukocyte protease inhibitor or even re-acidification) and may facilitate or complete HIV inactivation before infection can occur (Lai et al., 2009; Mascola and Montefiori, 2010). Additionally, virus which has been trapped and complexed by IgA will be naturally removed within cervical mucus.

In summary, PID's high sequence conservation was the decisive factor for the choice of the 3D6 and 4B3 antibodies. The rationale for isotype switching both antibodies from IgG to IgA was given due to the naturally protective role of IgA in mucosal tissues and its capability to aggregate antigen to form large immune complexes that could limit virus spread. Considering the rapidly rising incidence of new infections (Mestecky and Fultz, 1999) we hypothesized that an antibody vaccine which is a protection against HIV directly at the major port of entry could be beneficial. Ongoing and future experiments in a macaque model will prove if the isotype switched antibodies 3D6-IgA and 4B3-IgA can successfully aggregate virus, prevent it from passing through the mucosal layer and prevent infection during vaginal challenge with HIV-1.

1 Scientific Background

1.1 Introduction to the physiology of (recombinant) protein expression

Protein synthesis is a process fundamental to all cells that allows them to regulate intracellular processes, interact with their environment and secrete proteins as second messengers. The information, which is essential for the production of novel proteins, is ultimately stored in particular DNA nucleotide sequences, or genes. The respective genes need first to be transcribed into messenger RNA (mRNA) molecules, which can then be translated into protein entities. Both mRNA and protein molecules are processed and modified along the way. While prokaryotic cells perform all steps within the cytoplasm, eukaryotic cells comprise specialized compartments (i.e. organelles) for the respective reactions to take place. Newly produced proteins are either destined for the cytoplasm, for the organelles or are eventually secreted.

Gene expression is a highly regulated process and several mechanisms are in place to make sure that only the required amounts of the desired proteins are produced. Cells control protein synthesis by (1) controlling when and how often a given gene is transcribed (transcriptional control), (2) splicing/processing the primary RNA transcript (RNA processing control), (3) mRNA export from the nucleus in the cytoplasm (RNA transport control), (4) selection of mRNAs to be translated by ribosomes (translational control), (5) selective destabilization of particular mRNAs (mRNA degradation control), or (6) controlling the posttranslational activation, inactivation or compartmentalization of respective protein molecules (protein activity control) (Alberts et al., 2007) as outlined in Figure 1. It should be noted that the mechanism of prokaryotic protein expression is beyond the scope of this thesis.

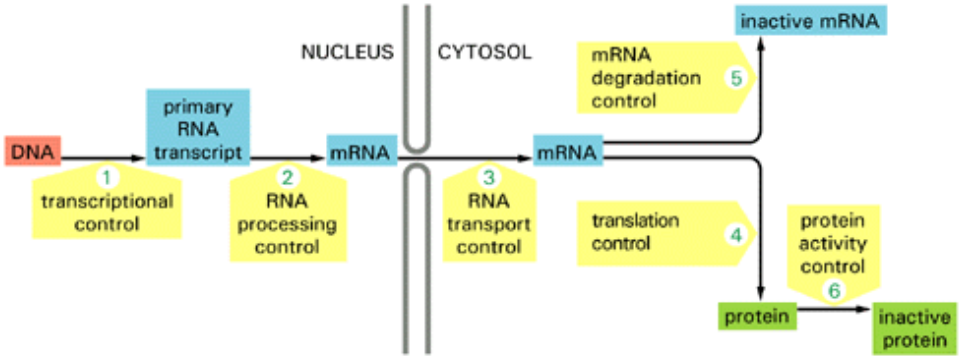

Figure 1. Six steps which allow control of eukaryotic gene expression (Alberts et al., 2007).

1.2 Description of the synthesis pathway

1.2.1 Transcription, mRNA modification and nuclear export

Transcription in eukaryotic cells is regulated by the composition and structure of DNA. DNA forms a double helix that is associated with histones and several other proteins to form a chromosome. The structure of the helix is governed by the nucleotide sequence, which provides a certain pattern that can be recognized by gene regulatory proteins and enables them to bind. The binding points provide hydrogen bond donors and acceptors that can be recognized by gene regulatory proteins and are located on the outside of the DNA double helix, mainly at the major groove.

Furthermore, there are short stretches of nucleotides (<20 bps) located on the DNA, which impact protein expression. Such sequences contain binding regions for gene regulatory proteins and therefore serve directly as genetic switches. Nucleotide sequences which augment protein expression upon binding of gene regulatory proteins are named enhancers; sequences that impede transcription are called silencers. The tightness of protein-DNA binding is essential for the function of gene regulatory proteins and is determined by ionic, hydrogen and hydrophobic interactions (Alberts et al., 2007).

Besides DNA structure and its regulative elements, transcription is also dependent on the structure of chromatin and the accessibility of a respective gene. Generally, there are two options: Genes can either be easily accessible as in *euchromatin* or the DNA can be tightly packed and associated with protein molecules to form compact structures as is the case in *heterochromatin*. In the latter case the coding regions are not easily tangible and gene expression is hampered. The chromosomal site of integration of a gene has a major effect on its transcription rate, which has led to calling this phenomenon “position effect”. Gene transcription can even be influenced by neighbouring heterochromatin (Chusainow et al., 2009).

Transcription cannot be initiated by RNA polymerase II alone but requires the additional assembly of so-called *general transcription factors* at the promoter. This assembly process is another method by which eukaryotic cells can regulate the rate of transcription initiation - either speeding it up or slowing it down. As mentioned above, the fate of transcription is determined by binding of gene regulatory proteins. Generally, there are two different categories of gene regulatory proteins: *transcriptional activators* also known as *gene activator proteins*, which support transcription and *transcriptional repressors* which inhibit transcription. A striking feature of gene regulatory proteins is that they can influence transcription upstream and downstream of a gene and from great distances even if they are thousands of nucleotides away from a promoter as shown in Figure 2. This is possible through e.g. an enhancer which binds proteins and later bends towards a

promoter region. In this way the proteins bound to the enhancer are able to interact with the general transcription factors or RNA polymerase II.

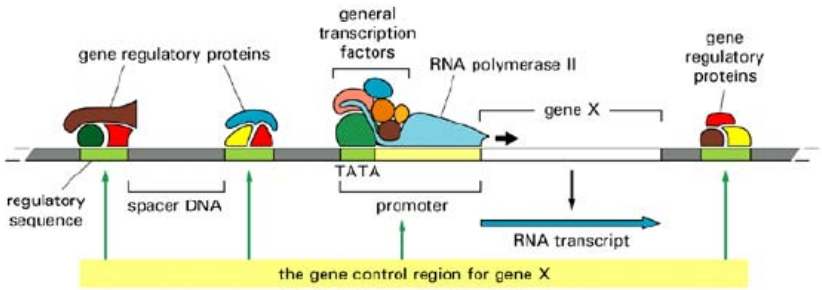

Figure 2. Different regulatory factors which control eukaryotic genes (Alberts et al., 2007).

Transcription is initiated by the binding of the transcription factor TFIID to the TATA sequence which typically is located 25 bps upstream of the transcription start site. Then, other general transcription factors as well as RNA polymerase II bind as shown in Figure 3. Upon binding of RNA polymerase II to the promoter it is activated by TFIIH and released from the general transcription factors to initiate transcription (Alberts et al., 2007). During transcription a segment of DNA is used as a template to copy the stored information and generate mRNA molecules.

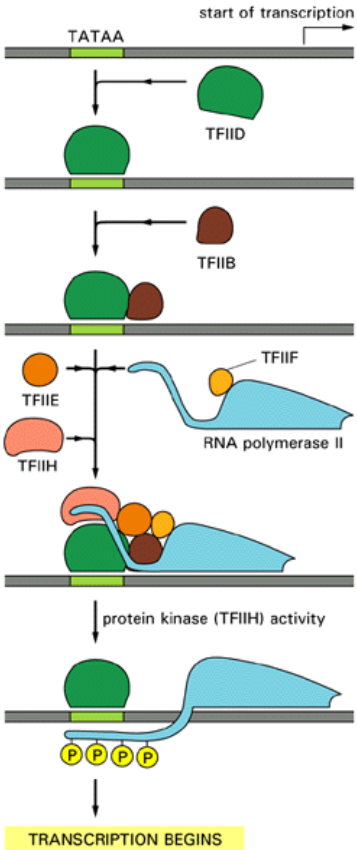

Figure 3. Transcription initiation by RNA polymerase II requires the assembly of general transcription factors (Alberts et al., 2007).

After the primary mRNA transcript has been produced, protein synthesis can still be regulated by processing and converting it into a mature mRNA molecule – ready for translation. Posttranscriptional controls include RNA splicing, capping, polyadenylation and mRNA editing. RNA splicing functions by removing the non-coding intron sequences and fusing the coding exon sequences together. Alternative RNA splicing allows a variety of polypeptides to be produced from the same gene. Capping involves the addition of a 7-methylguanosine residue to the mRNA 5'-end whereas the 3'-end is cleaved and subsequently polyadenylated. Both capping and polyadenylation protect the mRNA from nuclease digestion and are important signals for a mature mRNA to be exported from the nucleus to the cytoplasm (Alberts et al., 2007). RNA-editing allows for the insertion, deletion or substitution of nucleotides at particular points of the mRNA post transcription. This enables changes in the reading frame, hence in the sequence and leads to more diversity of the transcript.

1.2.2 Translation and post-translational modifications

The mature mRNA molecule is transported through the nuclear pore complex into the cytoplasm. Even in the cytoplasm gene expression can still be monitored via controlling the stability of mRNA.

During translation the encoded mRNA information is converted into a protein sequence. Translation requires association of the small ribosomal subunit loaded with an initiator tRNA as well as initiation factors (especially eukaryotic initiation factor 2, eIF-2) to the start codon. Then, the initiation factors will disassemble in exchange for the large ribosomal subunit and form a completed ribosomal complex composed of rRNA and protein. Ribosomes catalyze the translational process and must ensure the proper pairing of a tRNA anticodon with the respective mRNA codon as well as linking a tRNA's amino acid to the growing polypeptide chain. In eukaryotic cells a single mRNA is typically translated by multiple ribosomes (also known as polyribosomes or polysomes) at the same time. Protein synthesis continues until a stop codon is encountered, which causes special release factors to bind. This will terminate translation and leads to the dissociation of mRNA, ribosome and protein (Alberts et al., 2007).

Some proteins are entirely synthesized by free ribosomes in the cytoplasm. Proteins which are destined for cell surface expression or secretion (e.g. antibodies) will be processed by the endoplasmic reticulum (ER) which is a complex of flattened sacks and tubules with an essential role in protein and lipid synthesis. Proteins destined for modification in the ER contain a specific signal sequence at the N-terminus that is 5-30 amino acids in length and guides the proteins towards the proper locus.

Once the ER signal peptide is exposed from its synthesizing ribosome it is bound by the signal-recognition particle (SRP) as shown in Figure 4. This briefly arrests the translational process and allows the formed complex to bind to the SRP receptor on the cytosolic ER surface. In a current model it is suggested that the SRP is only released after the ribosome has adequately engaged with the translocon apparatus in the ER membrane (Alberts et al., 2007). The translocon itself is composed of Sec proteins, which associate into the Sec61 heterotrimer that enables the transport of the nascent polypeptide through the hydrophobic ER membrane. The import of proteins through the translocon apparatus into the ER requires ATP hydrolysis and occurs co-translationally i.e. while the polypeptide is being synthesized from the mRNA template. Recent data suggests that polypeptides are translocated across the ER membrane by a protein translocator that forms an open pore upon ribosomal binding and closes upon completion of protein synthesis and ribosome detachment (Alberts et al., 2007).

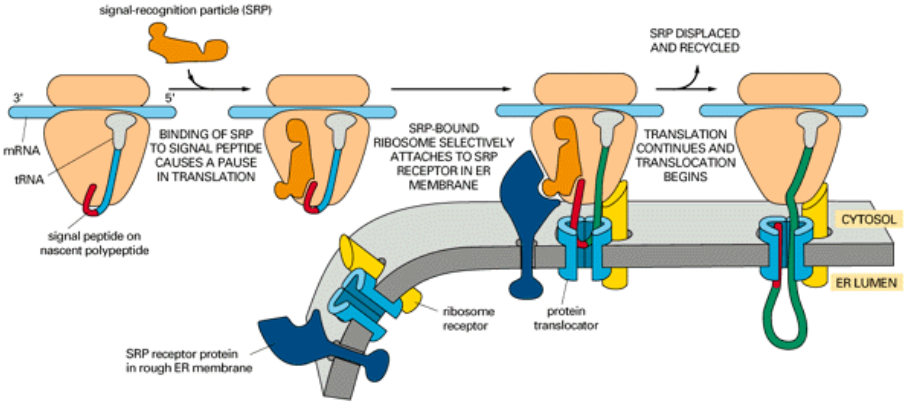

Figure 4. ER signal peptide recognition by SRP and direction to the ER membrane (Alberts et al., 2007).

Upon guiding the nascent polypeptide to the translocation apparatus the signal peptide is cleaved by an ER-resident signal peptidase while the synthesized protein is continuously being translocated into the ER lumen. It should be noted that the translocation of transmembrane proteins is a complex process which involves the folding back and forth of the growing polypeptide chain through the ER membrane via hydrophobic start and stop-transfer peptide anchors, but this is not being dealt with in this thesis.

The ER provides an environment which is optimized for protein folding and maturation. Several co-translational and post-translational modifications are carried out in the ER which do not take place in the cytoplasm. The present redox conditions in addition to the chaperone content (Ellgaard and Helenius, 2003) enable several modifications on polypeptides as outlined in Table 1. The action of post-translational modifications cannot be predicted fully, but often influence a protein's characteristics such as immunogenicity, activity, half life, receptor binding, protease susceptibility, secretion and solubility.

Table 1. A list of the major post-translational modifications on proteins in eukaryotic cells.

Protein modification	Function
Disulfide bonding	Protein disulfide isomerase (PDI) uses the oxidative environment in the ER to catalyze the oxidation of free sulfhydryl groups on cysteines to form disulfide bonds and therefore arrests proteins in a certain fold.
Protein folding	Binding protein (BiP) assists in protein folding by binding to incorrectly folded proteins and preventing exposed hydrophobic sequences to aggregate. Furthermore, only upon dissociation of BiP is a protein allowed to exit the ER. Calnexin and calreticulin only allow correctly folded and processed N-linked glycoproteins to exit the ER.
Oligomerization	Two or more polypeptide chains associate to form more complex structures.
Phosphorylation	Addition of phosphate to serine, threonine or tyrosine by a kinase. De-/Phosphorylation is a common mechanism to turn protein function(s) “on” and “off”.
Acetylation	Addition of acetyl groups by acetyltransferases influences protein activity (similar to de-/phosphorylation) and can also act as a signal for protein degradation.
N-linked glycosylation	Most soluble and membrane-bound proteins (unlike most cytosolic proteins) are glycosylated. During N-linked gylcosylation, oligosyl transferases which are bound to the luminal ER membrane catalyze the addition of lipid-linked precursor oligosaccharides from dolichol to asparagine residues.
O-linked glycosylation	O-linked glycosylation involves the transfer of oligosaccharides onto serine, threonine or hydroxylysine and occurs in the Golgi apparatus. Trimming and processing of the precursor oligosaccharides occurs in both the ER and Golgi apparatus.
GPI anchor	Addition of glycosylphosphatidylinositol (GPI) anchor to a protein’s C-terminus in the ER for membrane attachment.
Prenylation	Addition of a fatty acid chain or prenyl group (i.e. farnesyl by a farnesyltransferase or geranyl-geranyl by a geranyl-geranyltransferase I) to a protein’s C-terminal cysteine in the ER for cell membrane attachment.
Myristoylation	Addition of a myristotyl residue by N-myristoyltransferase to a protein’s N-terminus for cell membrane attachment.
Palmitoylation	Fatty acid addition to cysteine, serine and threonine by a palmitoyl protein thioesterase for membrane association.
Sulfation	In the Golgi, sulphate groups may be added to tyrosine residues by tyrosylprotein sulfotransferases which enables Ca^{2+} binding and influences a protein’s biologic activity.

Readily-processed proteins exit the ER into the Golgi apparatus via COPII (coatomer protein II) coated vesicles for further modifications. Chaperones possess a central role since they are involved in regulating the export from the ER by interacting with cargo proteins to support the folding pathway as well as preventing pre-mature export e.g. subunit proteins prior to their assembly into multimeric complexes. Additionally cargo-specific ligands are often involved in selectively binding cargo proteins, thereby altering their conformation and inducing export (Aridor and Balch, 1999). In order to ensure that chaperones remain in the ER they contain a retrieval signal composed of a KDEL (lysine, aspartate, glutamate, leucine) tetrapeptide. The KDEL receptor interacts with the KDEL signal to retain the chaperones and if necessary retrieves them from the Golgi apparatus via COPI coated vesicular transport (Ellgaard and Helenius, 2003).

The Golgi apparatus looks like a stack of plates and is composed of four to six flattened, membrane-bounded cisternea (Figure 5). Transport of proteins and lipids from the ER to the Golgi apparatus and *vice versa* as well as within the Golgi network is mediated by vesicular transport. Molecules enter the Golgi apparatus from the ER from the *cis* Golgi network whereas the exit site towards other cell compartments or cell surface for secreted molecules is termed *trans* Golgi network. The Golgi apparatus receives cargo from the ER by budding off of vesicles from a specialized region called the transitional elements that does not contain any ribosomes. Proteins are packed into these vesicles in a nonselective manner as long as they are correctly folded and assembled. Misfolded and misassembled proteins are retained in the ER, either bound by BiP, or in form of aggregates that cannot be packed and are subsequently degraded (Alberts et al., 2007).

In the Golgi apparatus the oligosaccharide chains of glycoproteins are further modified according to their accessibility by trimming and addition of further sugar residues. Each of the Golgi cisternae contains different sets of oligosaccharide processing enzymes. If a proteins' configuration excludes enzymes from accessing a sugar residue it typically results in high-mannose oligosaccharides. In this case no new sugars will be added and such proteins therefore only contain two N-acetylglucosamines as well as multiple mannose residues. Sugar residues which can be accessed easily by enzymes will be modified to complex oligosaccharides, which additionally contain alternating numbers of galactose, sialic acid and occasionally fucose. Readily processed glycoproteins will be forwarded by the *trans* Golgi network to lysosomes, secretory vesicles or the plasma membrane. During exocytosis vesicles that bud off from the *trans* Golgi network fuse with the plasma membrane to release their content into the extracellular space.

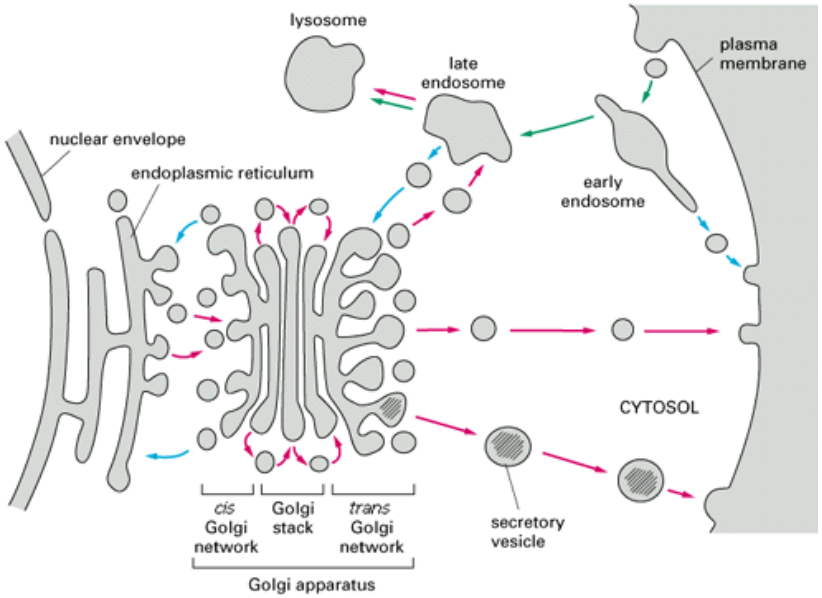

Figure 5. Vesicular transport of secretory and endocytic pathways employed by eukaryotic cells. The biosynthetic-secretory pathway (red arrows) transports protein molecules from the ER to the plasma membrane or (via late endosomes) to lysosomes. The endocytic pathway (green arrows) forwards molecules that are ingested in vesicles derived from the plasma membrane to early endosomes and then (via late endosomes) to lysosomes. Many endocytosed molecules are retrieved from early endosomes and returned to the cell surface for reuse; similarly, some molecules are retrieved from the late endosome and returned to the Golgi apparatus, and some are retrieved from the Golgi apparatus and returned to the ER. All of these retrieval pathways are shown with blue arrows (Alberts et al., 2007).

1.2.3 Protein aggregation, stress response and protein degradation

The ER quality control (ERQC) monitors if protein folding and assembly are correctly carried out. Furthermore, ERQC has a decisive role in preventing immature and irreversibly misfolded molecules from leaving the ER (Sayeed and Ng, 2005). This is highly essential assuming that up to 30% of all newly synthesized proteins are estimated to be defective (Schubert et al., 2000) and that incompletely folded proteins are potentially damaging to the cell (Ellgaard and Helenius, 2003). Erroneous proteins may arise from mistakes during transcription and translation, oligomeric assembly of multi-subunit proteins and/or environmental stress such as oxidative damage or critical temperatures (Ellgaard and Helenius, 2003). If a protein cannot reach a native conformation it will eventually be targeted for degradation via a pathway termed ER-associated degradation (ERAD).

Native and non-native conformations can be distinguished by molecular chaperones because of their inherent sensor function to specifically interact with exposed hydrophobic regions of incompletely folded proteins (Ellgaard and Helenius, 2003). Chaperones bind incompletely folded proteins and thereby assist in folding, stabilization and prevent aggregation. If proper protein folding is inhibited and aggregated protein

species accumulate in the ER the cell will activate a signal transduction network termed the unfolded protein response (UPR). The UPR is a response to stress situations when proteins cannot be folded adequately and aims to re-establish a cell's homeostasis (Tsai et al., 2002; Schröder, 2006). The accumulation of protein aggregates triggers the activation of ATF6 (activating transcription factor 6) to act as a transcription factor for the expression of ER chaperone genes (Shen et al., 2002). It seems likely that ATF6 is normally inhibited by BiP, but is set free upon ER stress due to the competitive binding of BiP for ATF6 and unfolded proteins (Ellgaard and Helenius, 2003). Strategies of UPR include the expression of ER resident chaperones and protein foldases to support the ER folding capacity, synthesis of phospholipids to increase the volume of the ER and dilute its content, attenuation of transcription of genes encoding secretory proteins, general translation attenuation, and up-regulation of ERAD to decrease the unfolded protein load of the ER, and eventually induction of apoptosis upon persisting ER stress (Schröder, 2006).

Stress signalling in the ER can also be triggered when excess cargo proteins accumulate due to the over expression of protein subunits that cannot assemble for export. This so-called "ER overload response" also leads to the induction of chaperone-encoding genes to up-regulate the folding capacity as well as blocking novel protein synthesis (Aridor and Balch, 1999).

One of the best understood pathways of protein folding in the ER is the calnexin/calreticulin cycle Figure 6. Upon tagging of the core oligosaccharide ($Glc_3Man_9GlcNAc_2$) onto the nascent polypeptide glucosidase I and II two glucose residues cleave to generate a monoglucosylated glycoprotein. Calnexin and calreticulin bind to this glycoprotein together with the thiol-disulphide oxidoreductase ERp57. After removal of the remaining glucose residue by glucosidase II, calnexin and calreticulin are released and the correctly folded protein exits the ER. If the native conformation cannot be obtained a single glucose residue will be placed back onto the glycoprotein (by UDP-glucose:glycoprotein glucosyltransferase) to re-initiate the calnexin/calreticulin cycle. Permanently misfolded proteins will be cleaved by ERα1,2-mannosidase I to remove mannose. Such glycoproteins will then be recognized by ER degradation-enhancing 1,2-mannosidase-like protein (EDEM) which probably targets them for ER-associated degradation (ERAD) (Ellgaard and Helenius, 2003).

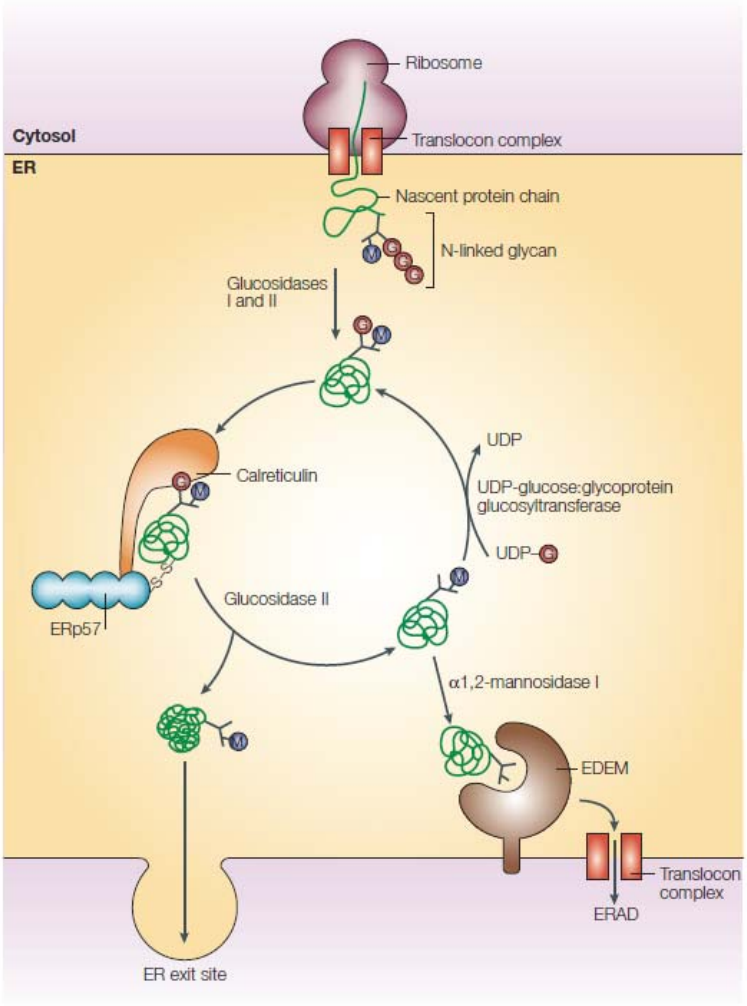

Figure 6. The calnexin/calreticulin cycle. For simplicity only calreticulin and only some sugar residues of the oligosaccharide core structure are depicted (Ellgaard and Helenius, 2003).

Since both proteins on the folding pathway and proteins destined for degradation expose hydrophobic regions it seems likely that the time of exposure allows the cell to distinguish between the two species. There is evidence that the timer is provided by ER mannosidase I which slowly catalyzes the mannosidase reaction and therefore only hopelessly misfolded proteins which occupy the ER machinery for extended periods will be trimmed. Upon binding of EDEM to the Man_8-species other chaperones are believed to be involved to start the retro-translocation of the glycoprotein into the cytoplasm for degradation. Among many potential candidates PDI appears to play an essential role in reducing disulphide bonds. BiP was found to be associated with immunoglobulin light chains before degradation and probably has a role to keep proteins in a non-aggregated state (Tsai et al., 2002).

ERAD requires retro-translocation of proteins destined for degradation from the ER into the cytosol. This is believed to occur via the translocon channel which is formed by the Sec61 complex. Proteins which undergo retro-translocation are polyubiquitinylated in the

cytosol via ubiquitin ligases upon their export from the ER. It is still a matter of debate whether polyubiquitinylation is a general feature of retro-translocation or not. Some substrates were determined to accumulate in the ER when polyubiquitinylation was prevented (Tsai et al., 2002). It was proposed that polyubiquitinylation might serve as a ratcheting mechanism to pull polypeptides into the cytosol and prevent it from moving back into the ER (Riezman, 1997). Additionally, ubiquitinylation might serve as a recognition signal for the proteasome (Tsai et al., 2002) which binds the polypeptide and then pulls it out into the cytoplasm (Mayer et al., 1998). In the cytosol proteins are degraded via the proteasome complex which possesses a cylindrical shape that is made up of multiple distinct proteases. Proteins destined for degradation are fed into the cylinder and are digested into short peptides (Alberts et al., 2007).

Protein aggregation in the ER is frequently irreversible and often associated with diseases. In fact, several serious diseases are known which are based on mutations and defects of endogeneous proteins that affect folding and subsequently lead to protein accumulation in the ER (Ellgaard and Helenius, 2003). In most diseases, so-called cargo coupling diseases, mutations of cargo molecules render them inaccessible for the ER transport machinery. Such mutations typically alter cargo folding, post-translational modification or oligomerization, or ligand interaction required for ER export (Aridor and Balch, 1999). As a consequence, such proteins are degraded to prevent their accumulation, however, their absence might trigger disease pathology. For example, overexpression of a mutant Δ508 cystic fibrosis transmembrane regulator that cannot be exported from the ER, due to the inability to engage the COPII machinery, is the causative of the most common form of cystic fibrosis (Kopito, 1999). Similarly, in hereditary hemochromatosis, which affects 5% of Caucasians, point mutations induce the loss of transferring receptor binding leading to degradation. If mutant cargo proteins additionally fail to be degraded and accumulate this can lead to chronic activation of ER stress responses which may trigger even more severe diseases. In cystic fibrosis the cystic fibrosis transmembrane regulator cannot be degraded efficiently which leads to the accumulation of ER or cytoplasmic aggregates and may induce the expression of NF-kB and inflammation. In inheritable peripheral neuropathies such as Perlizaeus-Merzbacher disease a chronic proteolipid accumulation leads to ER stress, apoptosis and defects in differentiation. In Alzheimer disease the ER processing is dysfunctional whereas unique conformational intermediates polymerize to form senile plaques (composed of amyloid ß-peptide generated through cleavage of a precursor protein) or tangles (abnormal aggregates of cytoskeletal tau protein) (Aridor and Balch, 1999).

It is assumed that degradation fails because the mutated proteins cannot achieve a proper intermediate conformation and therefore are masked from the degradation

machinery (Aridor and Balch, 1999). Other explanations why some aggregates cannot be exported from the ER include size limits that may have been exceeded, lack of mobility due to high protein concentrations that form a viscous, interconnected protein network and thereby hinder aggregates as well as smaller proteins to move to the ER exit sites (Ellgaard and Helenius, 2003). Heavily aggregated proteins seem to max out the ER quality control capacity which leads to the formation of non-degradable protein deposits known as Russell bodies (RBs). RBs were originally observed as aggregated immunoglobulins consisting of dilated ER cisternae that led to organelle deformations (Kopito and Sitia, 2000). It is believed that heavily aggregated proteins do not expose hydrophobic patches and therefore chaperones can no longer bind them. Similar to inclusion bodies they might compromise cell functions although not necessarily lead to cell death (Tsai et al., 2002).

A current model, the so-called N-end rule, suggests that in addition to the above mentioned criteria for protein degradation the first (N-terminal) amino acid in a polypeptide chain determines the stability of a protein. Amino acids such as Met, Ser, Thr, Ala, Val, Cys, Gly or Pro act in a stabilizing manner at the N-terminus of polypeptides in *S. cerevisiae*, whereas others stimulate protein degradation. In eukaryotes amino acids have different “potencies” in triggering the N-end rule: arginine is a primary, aspartate and glutamate are secondary, asparagine and glutamine are tertiary destabilizing amino acids (Alberts et al., 2007).

1.3 Recombinant antibody expression

1.3.1 Ig types and brief description

Antibodies or immunoglobulins are key mediators of the humoral immune response in the human body. Their specific mode of action can be transferred via “neutralization” of pathogens by specific association thereby preventing attachment to the target cell. Likewise, antibodies can bind toxins and thereby prevent their action. “Opsonization” allows antibodies to mark foreign structures in order to render them visible for the immune system and subsequently eliminate them either by phagocytes or “activation of the complement cascade”. The complement is also considered to be part of the humoral immune system. Antibodies are produced by B cells exclusively and are among the most abundant components in the blood accounting for up to 20% of total plasma protein (Alberts et al., 2007).

Antibodies have a Y-shaped structure with two identical antigen-binding sites, termed the Fab regions (fragment antigen binding), at the tip of each arm. Additionally, a hinge region makes the antibody arms flexible for interaction with target molecules. The

antibody tail is the effector or constant region, also called Fc region (fragment crystallizable), which is essential for the interaction with the immune system. An antibody monomer is composed of two identical light chains (LC) and two identical heavy chains (HC) which are assembled via a series of covalent and non-covalent interactions into globular structures, hence the term immunoglobulin. Each polypeptide chain possesses an N-terminal variable (V) and a C-terminal constant (C) region. The antibody's specificity for an antigen originates in the V region which consists of rather conserved framework regions as well as hypervariable or complementary determining regions (CDR) in-between. The C region of the HC determines the antibody isotype and is composed of several globular subunits which are homologues to one another and the C region of the LC (Alberts et al., 2007).

A complete antibody monomer is assembled out of a reservoir of one out of two LC classes (κ or λ) which are approximately 25 kDa in size. Additionally, there are five HC classes (α, γ, δ, ε, or μ) which possess a molecular weight of 50-60 kDa. The HCs differ in sequence, glycosylation and function and the HC sequence ultimately determines the antibody class. LCs can pair with any HC molecule, irrespective of the isotype. However, recent studies have suggested that there are naturally occurring preferences in VH/VL framework pairings with favourable biophysical properties (Tiller et al., 2013). In general, antibodies are categorized into five different classes: immunoglobulin A (IgA), immunoglobulin D (IgD), immunoglobulin E (IgE), immunoglobulin G (IgG) and immunoglobulin M (IgM). A more detailed overview of the basic features of the respective antibody classes is given in Table 2.

IgA is the second most prevalent antibody class in humans. There are two different subclasses (IgA1 and IgA2) which can exist as monomer in serum or form dimers in mucosal secretions. IgA possesses an additional 18 amino acid C-terminal sequence which allows the formation of polymeric molecules. Furthermore, incorporation of a third protein termed joining (J) chain enhances IgA polymerization and enables transcytosis via the polymeric immunoglobulin receptor (pIgR) into mucosal tissues to form secretory IgA (sIgA).

IgG is the main antibody class in humans and constitutes approximately 80% of total serum immunoglobulin. There are four different IgG subclasses (IgG1, IgG2, IgG3 and IgG4) which differ in their effector functions. IgGs are the major effector molecules of the secondary immune response and have a key role in activating the complement as well as binding macrophages and neutrophils.

IgD is mainly expressed on a B cell's surface and only up to 0.2% of total serum immunoglobulin is IgD.

IgE makes up the lowest fraction of immunoglobulin in the serum (0.005%). IgE binds to receptors on mast cells and basophils where antigen binding will trigger degranulation and cytokine secretion. Furthermore, IgE promotes allergic reactions and is involved in defense against parasites.

IgM is the first antibody class to be produced by a developing B cell. Similar to IgA, IgM contains an additional C-terminal segment which allows polymerization into large structures (pentamers and even hexamers) as well as incorporation of J chain.

Table 2. Basic features of the five human antibody classes.

Immunoglobulin	IgA	IgD	IgE	IgG	IgM
Heavy chain class and subclass	α α_1 α_2	δ δ_1 δ_2	ε	γ γ_1 γ_2 γ_3 γ_4	μ
Light chain type	κ and λ	κ and λ	κ and λ	κ and λ	κ and λ
Molecular weight (kDa)	160	180	200	150	900
Valence	2 or 4	2	2	2	10
Add. polypeptides	JC and SC	-	-	-	JC
Complement activation	-	-	-	+	+++
Plasma halflife (d)	6	3	2	23 7 for IgG_3	5-10
% total Ig	13	0.2	0.005	80	6
Serum conc. (mg/mL)	1.8	0.03	0.00005	10	1.2
Maternofetal transfer	-	-	-	+++	-
Antibacterial activity	++	-	-	+++	+++
Antiviral capacity	+++	-	-	+++	+
Allergic reaction	-	-	++	-	-
Antitoxin activity	+	-	-	+++	-
Agglutination capacity	++	-	-	+	+++
Presence in milk	+	-	-	+	Traces
Biological function	sIgA in mucosa	B cell de-velopment	Allergies, parasites	Secondary response	Primary response

1.4 IgA as an example for a highly complex protein

Immunoglobulin A is the second most prevalent antibody class in human serum (after IgG) and the most dominant antibody class in external secretions of mucosal tissues. Mucosal membranes include the linings of the respiratory, gastrointestinal and genitourinary tracts and make up a surface of about 400 m². The body spends considerable amounts of energy daily in order to produce IgA which protects against pathogens at this first line of defence.

1.4.1 Different forms of IgA

Similar to other immunoglobulines IgA is constructed by assembly of two identical light and heavy chains (Figure 7). In serum IgA mainly occurs as monomer, although dimeric (dIgA) or higher polymeric forms (pIgA) also exist. Although IgA possesses the inherent ability to form dimers (due to its heavy chain tailpiece), dimerization is enhanced by co-

expression of J chain in the antibody-producing plasma cell. Furthermore, J chain is essential for the transport of polymeric IgA into external secretions via pIgR. In external secretions a portion of pIgR remains attached to IgA, termed “secretory component” (SC), to form sIgA. sIgA predominantly appears as dimer in external secretions as well as higher polymerized isoforms.

In humans, IgA exists as either of the two subclasses IgA1 or IgA2. The two subclasses mainly differ in the length of the hinge region, which is extended by 13 amino acids in IgA1 (Woof and Kerr, 2006) and therefore enables it to have a more extended reach. IgA2 occurs in two allotypic variants, IgA2m(1) and IgA2m(2), however, a third allotype, IgA2n, may also exist (Chintalacharuvu et al., 1994). Remarkably, in IgA2m(1) there are no disulfide bonds linking LC and HC, instead the LCs are bound to each other. IgAs are heavily glycoslyated biomolecules whose molecular weight originates from N-glycosylation to 6-7% in IgA1 and 8-10% in IgA2 (Woof and Kerr, 2006). Interestingly, IgA1 additionally contains three to six O-linked glycosylation sites (Tarelli et al., 2004) in its hinge region.

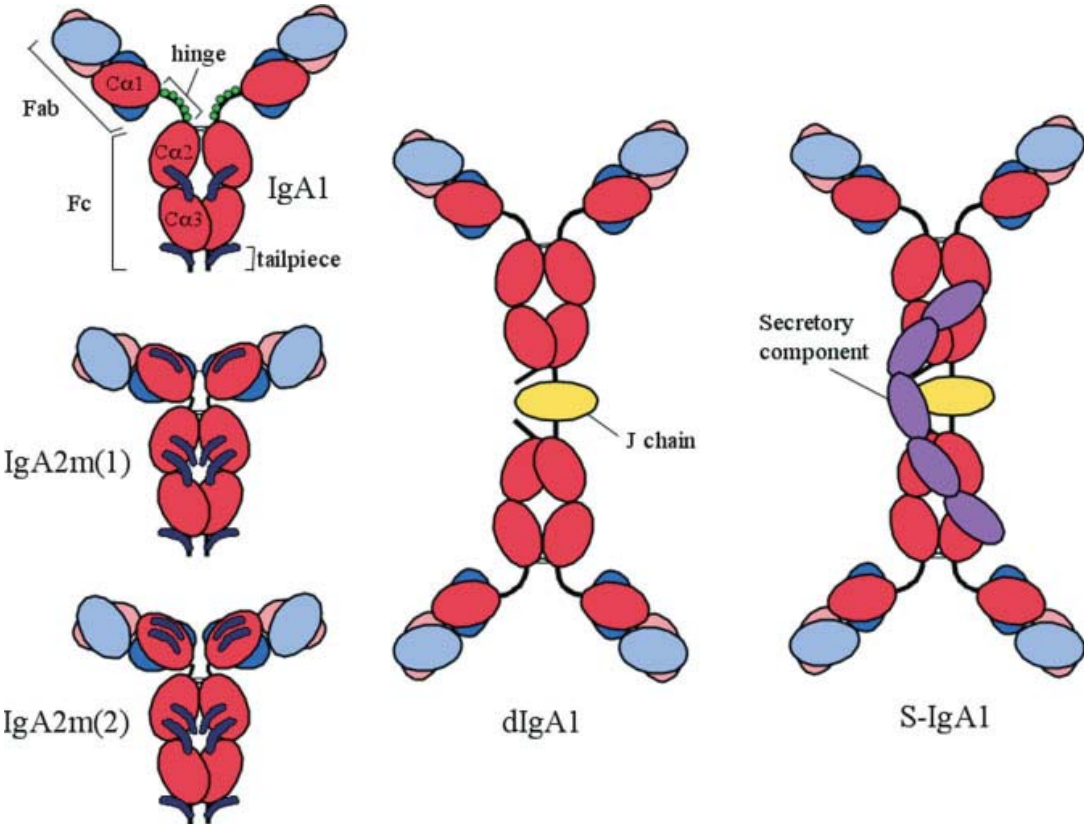

Figure 7. Schematic representation of monomeric IgA1, IgA2m(1) and IgA2m(2), as well as dimeric IgA1 and secretory IgA1. HC constant regions (red) and variable domains (pink) as well as LC constant regions (mid-blue) and variable regions (pale blue) are indicated. On the oligomeric forms of IgA, J chain is colored yellow and secretory component purple. On the IgA1 hinge region *O*-linked sugars are shown as green circles. *N*-linked sugar moieties are indicated in dark blue. it should be noted that for simplicity the oligosaccharide residues of dIgA and S-IgA1 are not included (Woof and Kerr, 2006).

Secreted IgA is mostly polymeric in nature, whereas dimeric forms dominate. In the latter case two IgA monomers are directly linked via a 15 kDa polypeptide termed J chain that connects the IgA tailpieces, an 18 amino acid C-terminal extension. Furthermore, sIgA contains an 80 kDa polypeptide termed secretory component (SC). SC corresponds to the extracellular part of the polymeric immunoglobulin receptor (pIgR) that remains attached to IgA upon its transcytosis into external secretions. While the attachment of SC is based on covalent and non-covalent interactions in sIgA1, it can solely be non-covalent in sIgA2 (Bonner et al., 2009b). During transcytosis, pIgA attaches onto pIgR, which is expressed at the basolateral surface of epithelial cells lining mucosal tissues, and subsequently endocytosed. Upon vesicular transport through the cell the pIgR-IgA complex is exposed at the apical surface where some as yet non-defined proteases cleave the extracellular pIgR portion to set the receptor-IgA complex free (Figure 8).

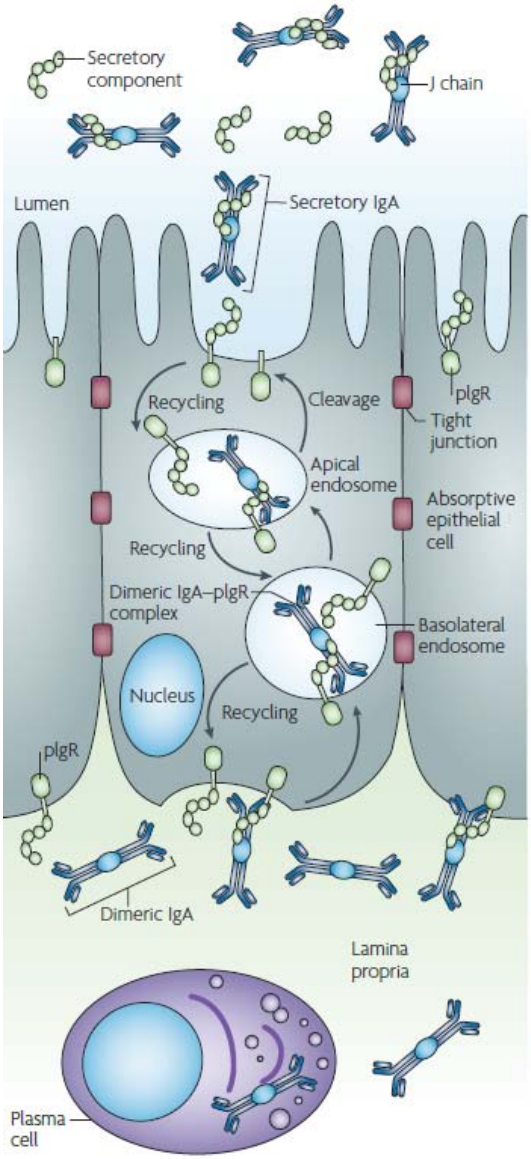

Figure 8. Transcytosis of polymeric IgA through pIgR mediated transport (Strugnell and Wijburg, 2010).

SC possesses inherent nonspecific antibacterial capacities itself and has several important functions such as selective transportation, localization and anchorage of sIgA as well as stabilizing the structure of sIgA and increasing its proteolytic resistance in mucosal tissues (Bonner et al., 2009a). The SC contains five Ig variable-type domains (D1-D5) which are 100-110 amino acid residues in length and 42 unstructured amino acids in-between for flexibility. A C-terminal linker region connects the SC complex to the membrane. Finally, SC contains seven putative N-linked glycosylation sites which enable a high glycosylation of up to 22% of its own molecular weight (Almogren et al., 2009).

1.4.2 Functions of secretory IgA

Secretory IgA is assumed to possess high capabilities in passive (and perhaps also active) immune protection due to inherently high concentrations in human colostrum and milk. In secretions of mucosal tissues the concentrations of complement proteins and leukocytes are rather low and therefore their immune effector functions are not as powerfully present as in internal tissues and fluids (Woof and Kerr, 2006). Secreted IgA protects mucosal surfaces from infections primarily through immune exclusion and intracellular neutralization and, to a lesser extent, through antigen excretion as indicated in Figure 9.

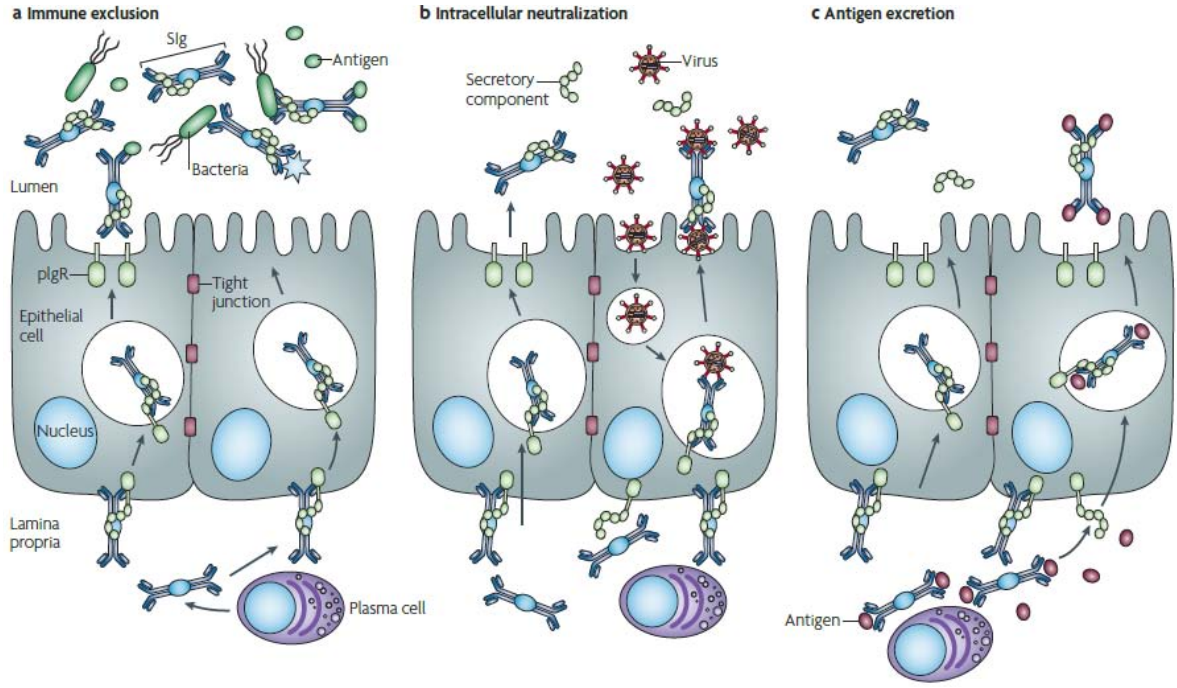

Figure 9. Three different functions by which secretory immunoglobulins protect mucosal surfaces (Strugnell and Wijburg, 2010).

Immune exclusion occurs when the contact between pathogens or toxins and mucosal epithelial cells is inhibited. As a result, harmful molecules cannot attach or invade epithelial cells which line mucosal tissues. Intracellular neutralization refers to the prevention of viral replication or toxin activity inside epithelial cells as a result of fusion of

sIgA-carrying endosomes with apically endocytosed virus or toxins. Specific interactions may inhibit virus replication (e.g. by preventing capsid removal) as well as preventing toxins from reaching their intracellular target receptor (Strugnell and Wijburg, 2010). In an experimental setup it has been shown that IgA added to the basolateral surface of polarized epithelial cells could neutralize intracellular Sendai virus replication, whereas this effect was absent when IgG was administered (Mazanec et al., 1992). Antigen excretion enables antigen removal from the apical epithelial surface by antigen binding of specific dIgA molecules and subsequent release at the luminal side mediated by pIgR. This was shown *in vitro* with antigen-specific IgA which allowed the excretion of whole measles virus by pIgR-mediated transport (Yan et al., 2002). Recently, the excretion of HIV by pIgR-facilitated transport has been shown (Wright et al., 2008).

The multivalency of sIgA is generally believed to facilitate aggregation, immobilization and neutralization of pathogens at mucosal surfaces. Naturally, dimeric IgA and other polymeric isoforms possess greater avidity to antigens than monomeric IgA itself. Upon encountering an antigen, the antigen-antibody complex can be eliminated from a mucous layer by dynamic processes such as peristalsis in the gastrointestinal tract and mucociliary movement in the airways (Corthésy, 2002).

2 Materials and methods

2.1 Generation of expression plasmids

Antibodies 3D6 (Grunow et al., 1988; Kunert et al., 1998) and 4B3 (Buchacher et al., 1994) were originally developed by hybridoma technology and isolated as IgG1 isotype. Isotype switching was performed by pasting the particular VH sequences into the constant region of an IgA1 heavy chain sequence. The cDNA sequence for human IgA1 heavy chain (J00220), kappa light chain (J00241) and lambda light chain (J00253) constant regions can be retrieved online via their given GenBank accession numbers. 3D6 and 4B3 light chain sequences remained unaltered. All cDNAs were codon optimized for expression in CHO cells and synthesized by GeneArt AG (Germany). The nucleotide sequences of all used plasmids and the amino acid sequences of the genes of interest are given in the appendix in section 10.1.

2.1.1 Generation of expression plasmids for polymeric IgA

For the expression of polymeric IgA, 3D6 and 4B3 IgA heavy chains were inserted into the plasmid pIRESdhfr_SV40 (Wolbank et al., 2003) under the control of a simian virus (SV) 40 promoter in combination with dihydrofolate reductase (dhfr) for gene amplification. 3D6 kappa light chain or 4B3 lambda light chain were cloned into a self assembled plasmid pSV40 containing the SV40 promoter and the SV40 polyA. The gene of the joining chain, which facilitates IgA polymerization, was introduced in the plasmid pIRESneo_SV40 (Wolbank et al., 2003) under control of the SV40 promoter and contained a neomycin cDNA. The plasmids expressing JC, LC and HC are called pIRESneo_JC, pMG433_3D6IgA_LC (or pMG433_4B3IgA_LC) and pIRESdhfr_SV40_3D6IgA_HC (or pIRESdhfr_SV40_4B3IgA_HC), respectively, and are shown in Figure 10.

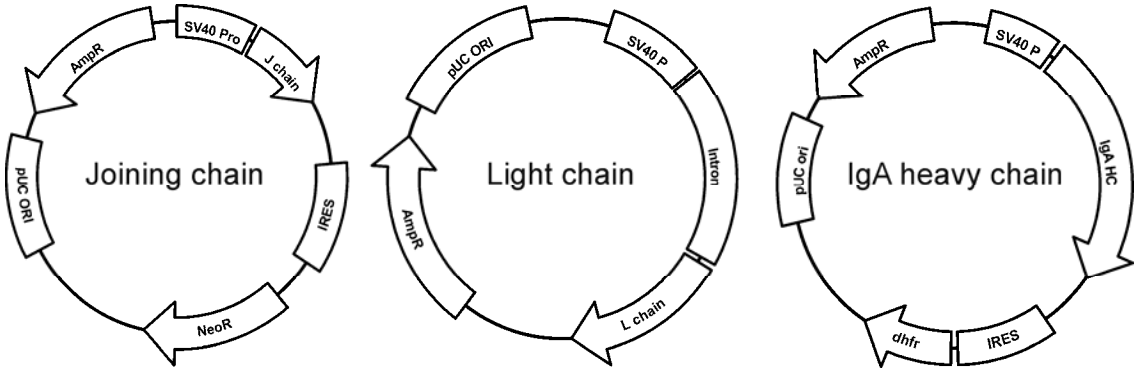

Figure 10. Plasmids used for the transfection of dhfr deficient CHO cells to produce human IgA.

2.1.2 Generation of expression plasmids for secretory IgA

For the expression of secretory IgA two cloning vectors were generated for the co-transfection of antibody heavy chain, light chain, J chain and human secretory component. Briefly, one plasmid (i.e. pIRES(AB)neo_LC_JC) was designed to contain the antibody light chain and J chain gene together with a neomycin resistance gene; a second plasmid (i.e. pIRES(AB)dhfr_HC_hSC) included the antibody heavy chain and hSC gene together with a dhfr gene.

The plasmid pIRES(AB)dhfr_HC_hSC was generated from the bicistronic vector pIRES(AB)neo (Clontech Laboratories, CA) which contains an IRES (internal ribosome entry site) sequence for the simultaneous expression of two proteins separately but from the same mRNA transcript. Two cloning sites are located upstream and downstream of the IRES sequence under the control of a CMV (cytomegalovirus) promoter. Additionally the plasmid contains an origin of replication as well as ampicillin and neomycin resistance genes for selection in bacterial and mammalian expression systems, respectively. Initially, the latter one was exchanged for a dhfr expression cassette by scission with the restriction enzyme ClaI (NEB, UK) according to the manufacturer's instructions. Since ClaI is sensitive to DNA methylation by dam or dcm methylase the plasmid was previously transformed into dam and dcm negative *E.coli* cells (NEB, UK), cultivated and subsequently purified by column purification using the Wizard®SV gel and PCR clean-up system (Promega, WI) according to the manufacturer's instructions. The dhfr gene was obtained by PCR amplification from the plasmid p2_dhfr. For this purpose primers were generated which contained an additional nucleotide overhang that included a ClaI restriction site (not shown). After PCR amplification the reaction mix was separated by agarose gel electrophoresis to verify the correct size of the amplicon. The respective band was then excised from the gel and again purified by column purification. After restriction digest of the amplicon with ClaI and a subsequent purification it was ligated with the vector backbone to generate the plasmid pIRES(AB)dhfr (Figure 11) using T4 DNA ligase (NEB, UK) according to the manufacturer's instructions.

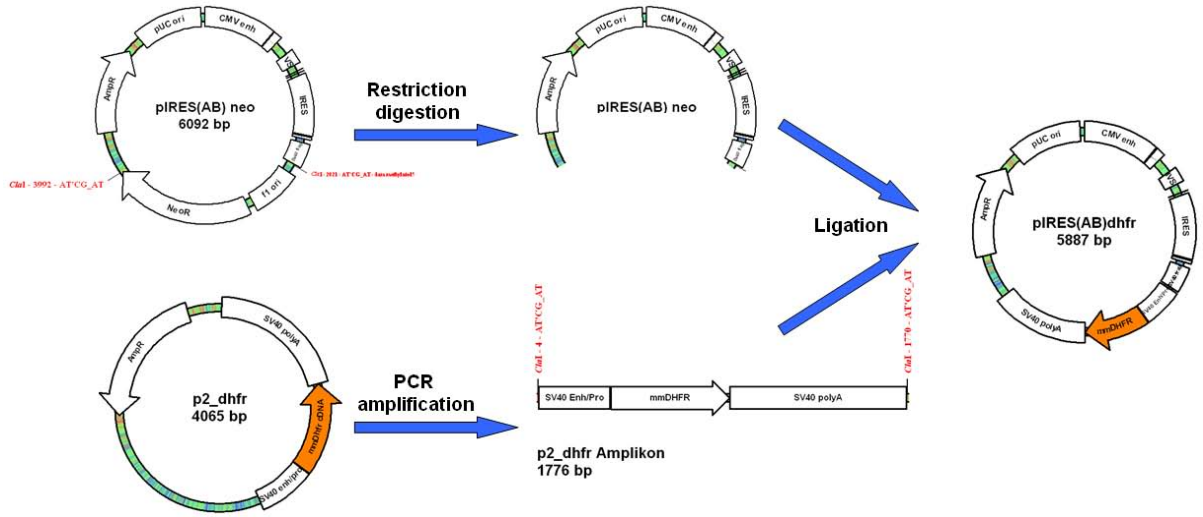

Figure 11. Cloning strategy for the generation of the plasmid pIRES(AB)dhfr.

Next, pIRES(AB)dhfr was digested with the restriction enzymes XbaI and NotI (both NEB, UK) in order to open the multiple cloning site B downstream of the IRES sequence motif. The same enzymes were used for the isolation of the hSC gene from the vector plasmid pCIneo_SC. By ligation of the hSC gene into the cut pIRES(AB)dhfr vector backbone the plasmid pIRES(AB)dhfr_hSC was generated (Figure 12).

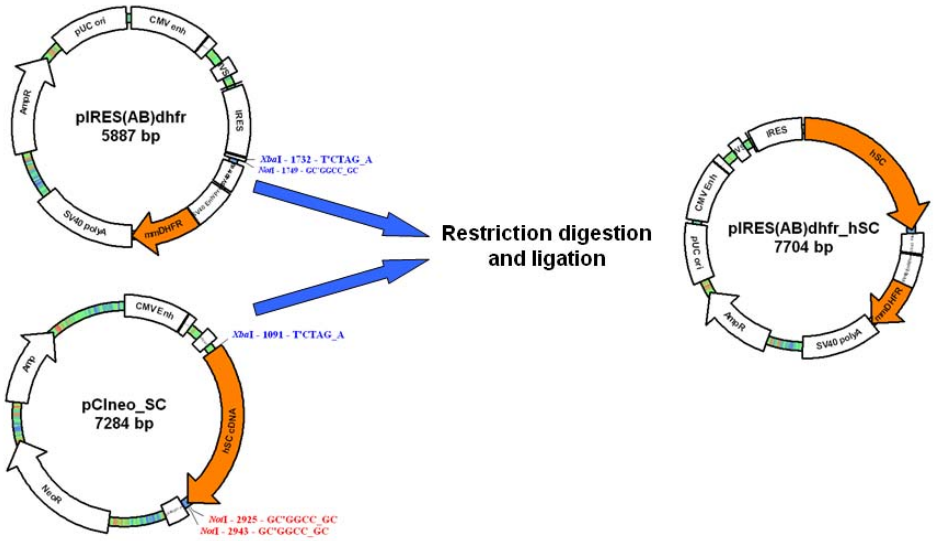

Figure 12. Cloning strategy for the generation of the plasmid pIRES(AB)dhfr_hSC.

In the last cloning steps the multiple cloning site A upstream of the IRES sequence of pIRES(AB)dhfr_hSC was cut open with the restriction enzymes EcoRI and KpnI (both NEB, UK). The same enzymes were used for the isolation of the HC gene from the vector plasmid pIRESdhfr_SV40_HC. By ligation of the HC gene into the cut pIRES(AB)dhfr_hSC vector backbone the plasmid pIRES(AB)dhfr_HC_hSC was generated (Figure 13).

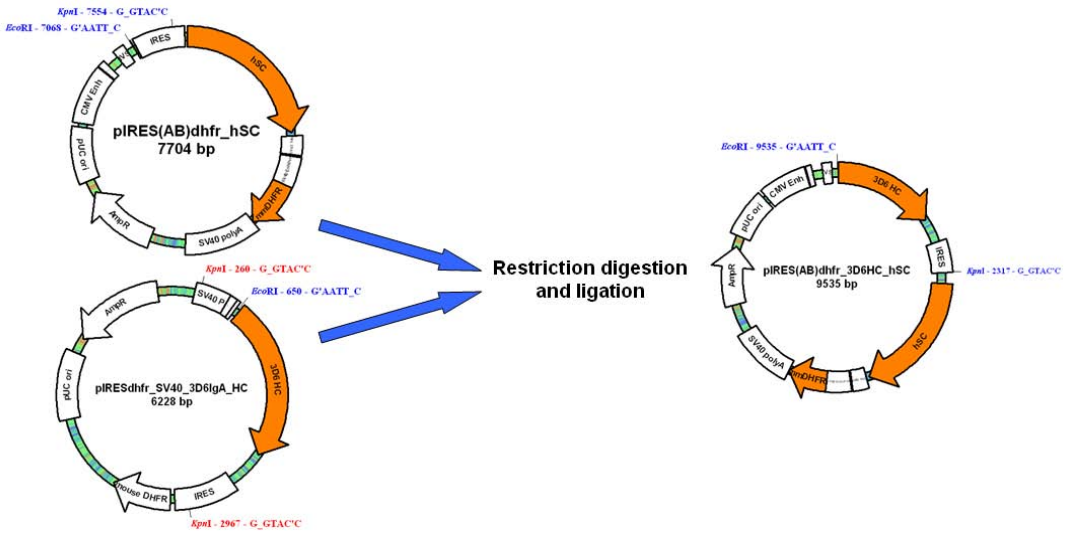

Figure 13. Cloning strategy for the generation of the plasmid pIRES(AB)dhfr_HC_hSC. It should be noted that although the given illustration only represents the cloning steps for 3D6 HC, 4B3 HC was identically cloned into the vector backbone.

The plasmid pIRES(AB)neo_LC_JC was generated from the above described vector pIRES(AB)neo by linearizing the vector and cutting it in the multiple cloning site B with the restriction enzymes XbaI and NotI. The J chain was obtained by cutting the plasmid pIRES(AB)neo_2G12-GL-LC_JC with the same restriction enzymes. Finally, the JC gene was ligated into the linearized backbone to create the plasmid pIRES(AB)neo_JC (Figure 14). It should be noted that directly using the plasmid pIRES(AB)neo_2G12-GL-LC_JC and solely cutting out the 2G12-GL-LC sequence was not possible due to the loss of necessary restriction sites as a result of prior cloning work.

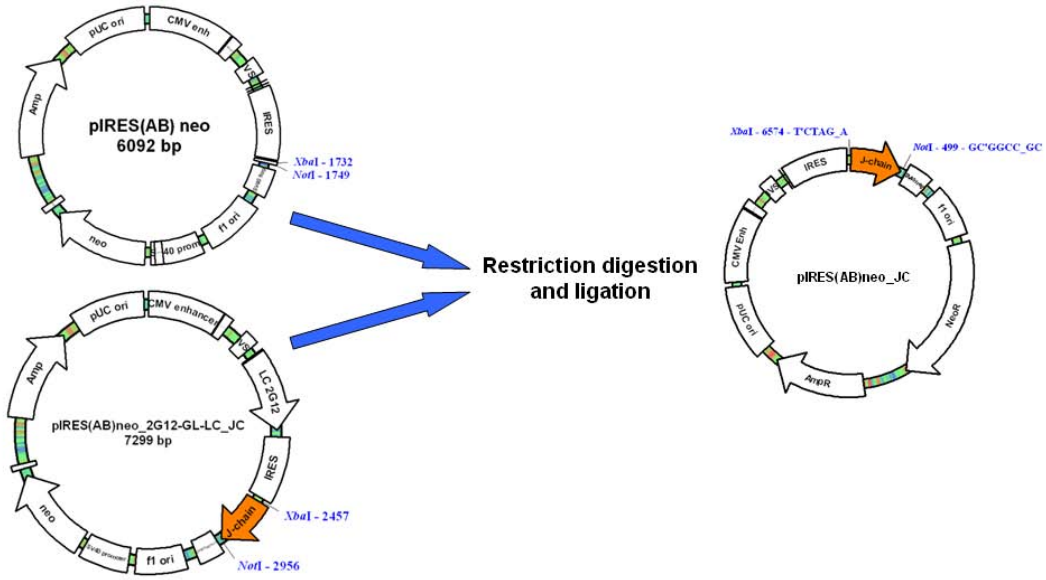

Figure 14. Cloning strategy for the generation of the plasmid pIRES(AB)neo_JC.

In the last cloning steps the multiple cloning site A upstream of the IRES sequence of pIRES(AB)neo_JC was cut open with the restriction enzymes EcoRI and MluI (both NEB,

UK). The same enzymes were used for the isolation of the LC gene from the vector plasmid pMG433_LC. By ligation of the LC gene into the cut pIRES(AB)neo_JC vector backbone the plasmid pIRES(AB)neo_LC_JC was generated.

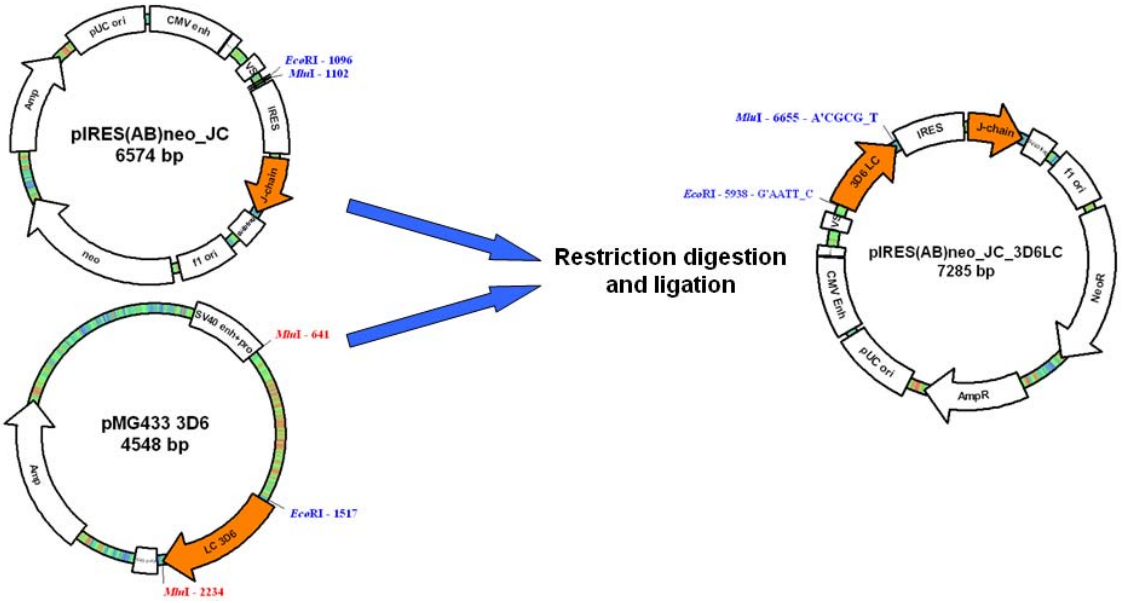

Figure 15. Cloning strategy for the generation of the plasmid pIRES(AB)neo_LC_JC. It should be noted that although the given illustration only represents the cloning steps for 3D6 LC, 4B3 LC was identically cloned into the vector backbone.

2.1.3 Generation of expression plasmids for human secretory component

For the generation of cell lines stably expressing hSC the plasmids shown in Figure 16 were used for co-transfection. The hSC gene was codon optimized for the expression in CHO cells, synthesized by Geneart and subsequently cloned in the multiple cloning site of the pCIneo expression vector using the NheI and SalI restriction sites up- and downstream of the gene of interest. The resulting vector pCIneo_hSC contained the coding sequence for human secretory component under the control of a CMV promoter together with a neomycin resistance gene for clone selection. Human secretory component represents the extracellular domain of pIgR which is cleaved by protease(s) which have not yet been characterized. Although the cleavage site may vary it has generally been agreed that hSC comprises the first 585 amino acids of pIgR (Bonner et al., 2007; Almogren et al., 2009). Therefore, we decided to synthesize hSC accordingly to contain only the first 585 amino acid residues of the pIgR upstream of the cleavage site. The second plasmid (p2_dhfr) contained a dhfr gene under the control of a SV40 promoter and was used for the purpose of gene amplification.

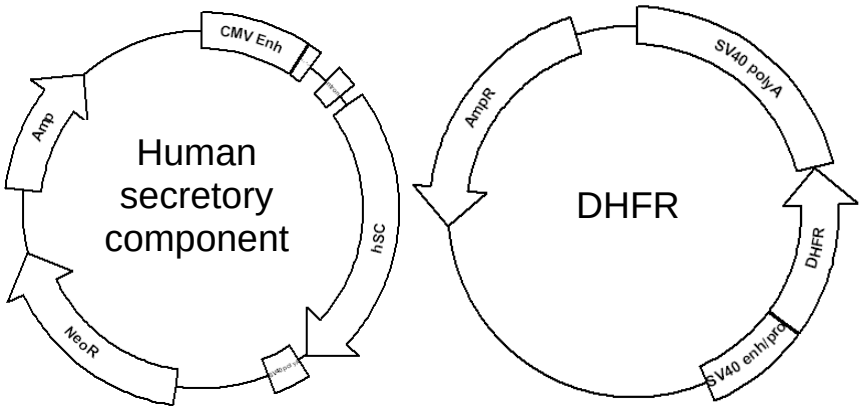

Figure 16. Plasmids used for the transfection of dhfr deficient CHO cells to produce hSC.

2.2 Generation of stable recombinant cell lines

The mammalian host cell line applied for stable recombinant mAb expression was dhfr deficient CHO cells, ATCC CRL-9096 (Urlaub and Chasin, 1980). These cells were grown under protein-free conditions in a cultivation medium consisting of a DMEM/Ham's F12 1:1 (PAA, Austria) formulation supplemented with 4 mM L-glutamine, 0.1 mM hypoxanthine, 0.016 mM thymidine, 0.25 g/l soy peptone, 0.1 % Pluronic F-68 and a protein-free supplement (Polymun Scientific GmbH, Austria).

Stable recombinant CHO cell lines expressing human IgA were generated by co-transfection of three different plasmids applying joining chain, light chain and heavy chain at a ratio of 1:1:1. Recombinant sIgA expressing cell lines were generated by co-transfection of two different plasmids at a ratio of 1:1. Cell lines expressing hSC were co-transfected with the plasmids containing hSC and dhfr at a ratio of 5:1.

Gene delivery was conducted using the cationic polymer polyethyleneimine (PEI) applying a modified protocol (Reisinger et al., 2009). Briefly, in each transfection experiment 5×10^6 cells were centrifuged at 188×g and subsequently resuspended in 10 ml transfection medium consisting of ProCHO5 (Lonza, Switzerland) supplemented with 4 mM L-glutamine, 15 mg/l phenol red, 0.1 mM hypoxanthine and 0.016 mM thymidine. PEI polyplexes were formed by incubation of 250 µg PEI (linear, MW: 25,000; Polysciences Inc., PA) with 25 µg plasmid DNA in 1 ml HBS buffer, pH 7.5 for 10 minutes at room temperature. Afterwards, the DNA/PEI polyplexes were added to the cell suspension and incubated at 37 °C, 7 % CO_2 for 4 hours. Then, a medium exchange was conducted. After 24 hours, the medium was replaced with 50 ml selection medium composed of ProCHO5 supplemented with 4 mM L-glutamine, 15 mg/l phenol red and 0.5 mg/ml G418. Subsequently, 100 µl cell suspension per well was seeded in five 96-well plates. In two transfection experiments a total of 960 wells were generated for each

IgA variant (3D6-IgA, 3D6-sIgA, 4B3-IgA, 4B3-sIgA). For the establishment of hSC expressing clones one reaction experiment with a total of 480 wells was performed.

Clones appeared 10-14 days post transfection and were fed with amplification medium consisting of 0.05 µM methotrexate (MTX) in selection medium. Antibody and hSC production of transfectants was determined by double sandwich ELISA as described below. The best 5 % of the producing wells were adapted to grow at 0.1 µM MTX. In two additional selection procedures, finally cells from 4 different wells were selected and seeded into spinner vessels (Techne, United Kingdom) to compare their viability, growth and stability of product expression for at least 10 passages.

For 3D6-IgA and 4B3-IgA, the best performing clone was further subcloned by limited dilution as described above to obtain a monoclonal cell line. For this purpose the MTX concentration was further increased to 0.4 µM in amplification medium. The highest expressing clones were selected over the following three passages. The 4 best clones were monitored according to the above described parameters for at least 10 passages. Then, the best clone was subcloned a third time using the previous approach.

2.3 Propagation of recombinant cell lines

Antibody-containing supernatants were generated by cultivation of recombinant cell lines at 37 °C in 50 ml spinner vessels at 50 rpm in amplification medium with 0.4 µM MTX. The same conditions were used for the production of hSC and sIgA, however, the amplification medium contained only 0.1 µM MTX. Cells were split every 3-4 days and re-seeded at 2.5×10^5 cells/ml.

Recombinant cell lines were cultured for at least 10 passages to monitor growth rate and stability of product expression. The specific productivities (q_P) were calculated as picogram mAb per cell per day (pcd) from the initial and final antibody titers T_0 and T_1 (µg/ml), respectively. The respective formula is given below.

$$q_P = \frac{(T_1 - T_0)\times \mu \times 10^6}{x_1 - x_0}$$

µ was calculated from the cell seeding concentration x_0 (cells/ml) and the cell concentration x_1 (cells/ml) finally achieved after time t (days) in culture prior to the next passage, as shown by the formula below.

$$\mu = \frac{\ln(x_1 / x_0)}{t}$$

2.4 Determination of cell concentration and viability

For the determination of the cell concentration the cell suspension was centrifuged at 188×g. The pellet was subsequently re-suspended in cell lysis buffer containing 0.1 M citric acid and 2% triton X-100. After an incubation period of at least 1 hour the total cell concentration was quantified by measurement of cell nuclei using a Coulter Counter Z2 (Beckman Coulter, CA) according to the manufacturer's instructions.

The viability of cell cultures was determined by trypan blue vital staining using a 0.4% trypan blue solution (Sigma, MO) and a Neubauer improved chamber (Laboroptik, UK) according to the manufacturer's instructions.

2.5 Product quantification by ELISA

2.5.1 Polymeric IgA ELISA

Antibody concentrations in culture supernatants were quantified by ELISA. Briefly, immunosorbent plates (Maxisorb 96 Well, Nunc) were coated with a goat anti-human IgA α-chain specific antiserum (Sigma, MO). IgA standard was obtained by 3D6-IgA and 4B3-IgA purification by CaptureSelect human IgA chromatography as described in the materials and methods section 2.12. The standard was quantified spectrophotometrically at 280 nm with the extinction coefficients of 1.217 (3D6-IgA) and 1.115 (4B3-IgA) and subsequently adjusted to a starting concentration of 400 ng/ml. Standard and supernatants were diluted in 1:2 series and subsequently coated onto microtiter plates. IgA product was detected applying a mixture of a goat anti-human κ-chain specific antiserum (Sigma, MO) and a mouse anti-human λ-chain specific antiserum (Southern Biotech, AL), both peroxidase-conjugated. Sample staining was conducted with ortho-phenylenediamine dihydrochloride. Finally, mAb titers were quantified using an Infinite M1000 microplate reader (Tecan, Switzerland) at a wavelength of 492 nm and a reference wavelength of 620 nm.

2.5.2 Secretory IgA ELISA

Secretory IgA was quantified using a similar protocol as described above. However, the standard was IgA from human colostrum (Sigma, MO) which was adjusted to a starting concentration of 1000 ng/mL. The microtiter plates were coated as described above. Samples were detected using a combination of mouse anti-human secretory component antiserum (Sigma, MO) and an HRP-conjugated anti-mouse-γ-chain antiserum (Sigma, MO).

2.5.3 Human secretory component ELISA

Quantification of secretory component was conducted using a sheep anti-human IgA secretory component antiserum (AbD Serotec, UK) for product capture. Detection was performed with the same antibody pair as described above for sIgA quantification. The standard was a kind gift from Dr. Armelle Cuvillier from B-Cell Design (France). The received standard was recombinantly produced with a polyhistidine tag and purified via affinity chromatography. For ELISA the secretory component was adjusted to a concentration of 1000 ng/mL.

2.6 Preparation of genomic DNA and cDNA

Genomic DNA (gDNA) was isolated from 2 x 10^6 cells using the DNA Blood Mini Kit (Qiagen, Netherlands) according to the manufacturer's instructions. The concentration of isolated gDNA was quantified spectrophotometrically at an absorbance of 260 nm and the purity was determined measuring the ratio at 260 nm and 280 nm. gDNA samples were stored at 4°C.

Cellular RNA was isolated from 5 x 10^6 cells using Ambion Tri Reagent Solution (Life Technologies, CA) according to the manufacturer's instructions. To remove DNA contaminations from extracted RNA the preparation was digested with 3 U DNase I (Qiagen, Netherlands) for 30 min at room temperature together with 160 U RNase inhibitor (Life Technologies, CA) and then inactivated for 10 min at 75 °C before another RNA precipitation step. Purified total RNA was dissolved in 25 µl RNase free water containing 60 U RNase inhibitor.

cDNA was obtained by reverse transcription. 1.5 µg RNA, 1µg random primers (Promega, WI) and 12.5 nmol dNTPs (New England Biolabs, MA) were incubated in a reaction volume of 14 µl for 5 min at 70 °C and 2 min at room temperature. Then, 40 U RNase inhibitor, 200 U M-MLV reverse transcriptase and buffer (both Promega, WI) were added to a reaction volume of 20 µl and incubated for 30 min at 37 °C before denaturation for 5 min at 95 °C.

2.7 Real-time PCR

Real-time PCR (qPCR) analysis was performed on a MiniOpticon qPCR device (Biorad, CA). Primers and the fluorogenic hydrolysis probes were synthesized by Sigma (MO). The same primers and probes were used for the analysis of gDNA and cDNA. The reaction mix included iQ Supermix (Biorad, CA), 6 pmol primer and 4 pmol hydrolysis probe for HC, JC and ß-actin quantification or 12 pmol primer and 8 pmol hydrolysis probe for LC determination in 20 µl reaction volume. 3 ng pre-denatured (99 °C, 10 min)

gDNA or 3 µL cDNA from a 1:50 dilution of the reverse transcription reaction was used directly for qPCR. Negative controls (NC), no template controls (NTC) and no reverse transcriptase controls (NRT) for transcript analysis were included in each run. The quantification cycle (Cq) was determined by linear regression and baseline subtraction using the CFX Manager (Biorad, CA). The mean qPCR efficiencies for HC, LC, JC and ß-actin were calculated from raw fluorescence data using the LinRegPCR software application, V12.17 (Ramakers et al., 2003; Ruijter et al., 2009; Tuomi et al., 2010). Quantification was done by relative quantification with efficiency correction (Pfaffl, 2001) using ß-actin as internal reference and expressed as ratios.

2.8 Flow cytometry

Cells were prepared for flow cytometry based on an established protocol (Borth et al., 2005). Briefly, 1×10^6 cells were washed with PBS (PAA, Austria) and then fixed in 1 ml ethanol (70 %) for 20 min at 4°C. After washing twice in 1 ml Tris buffer (0.1 M Tris HCl, 2 mM $MgCl_2$, pH 7.4) cells were blocked with 20% FCS in Tris buffer for 30 min at 37°C. Cells were then incubated for 30 min at 37°C with 200 µl Tris/FCS containing an Alexa-Fluor-488-conjugated goat anti-human IgA antiserum (Dianova, Germany) and a biotinylated goat anti-human λ-chain (Novus Biologicals, CO) or κ-chain (Agrisera, Sweden) antiserum. Cells were washed and incubated in 200 µl Tris/FCS with a Alexa-Fluor-647-streptavidin conjugate (Life Technologies, CA) for 30 min at 37°C and re-suspended in 500 µl Tris buffer with 50 ng/ml DAPI (Sigma, MO) for analysis. ER-specific proteins were analyzed using a DyLight-488-conjugated mouse anti-KDEL antiserum (Enzo Life Sciences, Austria). In each experiment 10,000 cells were analyzed using a Gallios Flow Cytometer (Beckman Coulter, CA) at the wavelengths of 488 nm, 638 nm or 405 nm. The fluorescence emissions were measured with a 525/40 BP filter (Alexa-Fluor-488; DyLight-488), a 660/20 BP filter (Alexa-Fluor-647) or a 450/40 BP filter (DAPI; ER tracker Blue-White DPX). Non-producing CHO host cells were used as negative controls.

2.9 Immunofluorescence microscopy

Cells were prepared by ethanol fixation and stained using the same antibody conjugates and protocol as described for flow cytometry. 30 µl of the stained cell suspension was transferred onto microscope adhesion slides (Marienfeld-Superior, Germany) and incubated for 10 min. Cells were washed three times with PBS and prepared for microscopy using the SlowFade Antifade Kit (Life Technologies, CA) according to the manufacturer's instructions. Four channel confocal images were taken with a Leica TCS SP5 II laser scanning microscope (Leica Microsystems, Germany) using sequential excitation at the wavelengths of 488 nm and 633 nm. Fluorescence of Alexa-Fluor-488

and Alexa-Fluor-647 was detected in the range of 500 nm - 552 nm and 690 nm - 750 nm, respectively. The microscope hardware setting was kept constant for imaging the wild type and the recombinant cells. Image processing was performed with Leica LAS AF and ImageJ software.

2.10 Immunoblotting of intracellular and secreted product

Intracellular protein was harvested by cell lysis using RIPA buffer (Sigma, MO) according to the manufacturer's instructions and the cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Switzerland) to avoid product degradation. Both the obtained extract and the secreted product were denatured in NuPAGE LDS 4× sample buffer (Life Technologies, CA), electrophoretically separated on NuPAGE Novex 3-8 % Tris-Acetate gels (Life Technologies, CA) and analyzed on Western blots applying the same antibodies as used in ELISA. It should be noted that the IgA standard was originally derived from human colostrum and therefore is of sIgA type.

2.11 Antibody characterization by SDS-PAGE

Selected samples of recombinant human IgA were separated on NuPAGE Novex 3-8 % Tris-Acetate gels (Life Technologies, CA). 3D6-IgA crude culture supernatants or 4B3-IgA concentrates were incubated in NuPAGE LDS 4× sample buffer (Life Technologies, CA) and separated for 60 minutes at 150 V on the gel (Vorauer-Uhl et al., 2010) together with HiMark Pre-stained High Molecular Weight Protein Standard (Life Technologies, CA) consisting of 9 protein bands in the range of 30-460 kDa. Afterwards, the gels were silver stained (Heukeshoven, 1985) for determination of IgA purity. IgA isoforms were analyzed by Western blotting of Tris-Acetate gels onto PVDF membranes (Millipore, MA). Heavy chains were detected by incubation with a peroxidase-conjugated goat anti-human IgA α-chain specific antiserum (Sigma, MO), light chains of mAb 3D6-IgA and 4B3-IgA were detected via the same antisera as applied in ELISA. The presence of the joining chain was verified via a rabbit anti-human joining chain antiserum (Antibodies-online, Germany) and a peroxidase-conjugated anti-rabbit IgG polyclonal serum (Sigma, MO). Finally immunoblots were detected with a luminol and peroxide reagent formulation (ECL Western Blotting Substrate; Thermo Fisher Scientific, MA) using a Fusion FX7 (PEQLAB Biotechnologie, Germany) chemiluminescence imaging system.

2.12 Antibody purification

Purification of recombinant IgA was investigated by two different protocols. The first approach was termed "Multiple step chromatography" and comprised several different chromatographic techniques to remove impurities from the target product. In an

alternative approach, termed “Single step chromatography”, a novel IgA affinity matrix was investigated to purify recombinant IgA by single step affinity chromatography. All chromatographies were performed on an ÄKTApurifier (GE Healthcare, United Kingdom) fast performance liquid chromatography system operated by Unicorn 4.11 software (GE Healthcare, UK). The flow rate was held constant at 0.5 ml/min for all chromatographic steps.

For antibody purification, cell suspensions were first clarified from cells and cell debris by centrifugation at 2000 rpm for 10 minutes. Collected supernatants were 0.22 µm filtered using Stericup vacuum filtration units (Millipore, MA). Supernatants were concentrated to 0.5 mg mAb per ml and 7× buffer exchanged against PBS, pH 7.4 by ultrafiltration/ diafiltration (UF/DF) applying a lab-scaled TFF system (Millipore, MA) equipped with a Kvick Start cassette (GE Healthcare, United Kingdom) with a molecular weight cut-off of 30 kDa.

Immobilized jacalin (Thermo Fisher Scientific, MA) was used in a primary antibody capture step. For this purpose, HR16/5 columns (GE Healthcare, United Kingdom) were filled with 3.2 ml resin material according to the manufacturer’s instructions. The column was equilibrated with PBS, pH 7.4 before loading the IgA-containing UF/DF retentate. A washing step with equilibration buffer was introduced to remove unbound protein before product elution with 1.5 M α-D-galactose in PBS, pH 7.4. The flow rate was held constant for all chromatographic steps at 0.5 ml/min.

Collected IgA was further purified by AEX using DEAE Sepharose Fast Flow (GE Healthcare, United Kingdom). Consequently, 3.3 ml of stationary phase was packed into HR 16/5 columns (GE Healthcare, United Kingdom). Recovered eluate from the lectin-based resin was 7× buffer exchanged against AEX equilibration buffer (20 mM Tris, 10 mM NaCl, pH 8.5), as described above. The column was equilibrated/washed with AEX equilibration buffer before and after retentate was applied onto the resin. A second washing step with 20 mM Tris, 100 mM NaCl, pH 8.5 was conducted prior to elution with AEX elution buffer (20 mM Tris, 200 mM NaCl, pH 8.5). Equilibration, washing and elution were performed at 0.5 ml/min.

Non-product related impurities in AEX eluates were precipitated by addition of 3 M $(NH_4)_2SO_4$, pH 8.5 to a final concentration of 1 M or 0.75 M $(NH_4)_2SO_4$ for mAb 3D6-IgA or 4B3-IgA, respectively. Prepared samples were then purified by HIC. A Tricorn 5/100 column (GE Healthcare, United Kingdom) was packed with 1.9 ml Phenyl Sepharose FF low sub (GE Healthcare, United Kingdom) and conditioned with HIC equilibration buffer (1 M or 0.75 M $(NH_4)_2SO_4$ in 20 mM Tris, pH 7.5). After sample application and washing with 0.5 M $(NH_4)_2SO_4$, pH 7.5 for mAb 3D6-IgA or 0.2 M $(NH_4)_2SO_4$, pH 7.5 for mAb 4B3-

IgA the product was eluted at 0.3 M or without $(NH_4)_2SO_4$, pH 7.5, respectively. The applied flow rate for all steps was 0.3 ml/min.

In the second approach, VHH chromatography was investigated using the CaptureSelect human IgA affinity matrix (BAC, Netherlands). The affinity matrix contains a 13 kDa llama antibody fragment which recognizes human IgA. A Tricorn 5/100 column was packed with 0.6 ml resin material and subsequently equilibrated with PBS, pH 7.4. Culture supernatants were centrifuged, 0.2 µm filtered and buffer exchanged against PBS, pH 7.4 as described above. After sample loading of UF/DF retentate the column was washed with equilibration buffer. Elution of IgA was performed with 0.1 M glycine, pH 2.0. The recovered product fraction was immediately neutralized to pH 7.5 with 1M Tris buffer (pH 8.5).

3 Results

3.1 Recombinant expression of human IgA

In the first screening round of transfectants the best 5 % of the producing wells (48 wells for each antibody variant) were selected. The titers in these 48 selected wells were in a comparable range for 3D6-IgA or 4B3-IgA subclones, but substantially differed between the two antibody variants as shown in Figure 17. Titers of 4B3-IgA subclones were mostly in the range of 10-100 ng/ml, whereas subclones expressing 3D6-IgA primarily ranged between 6,000-10,000 ng/ml.

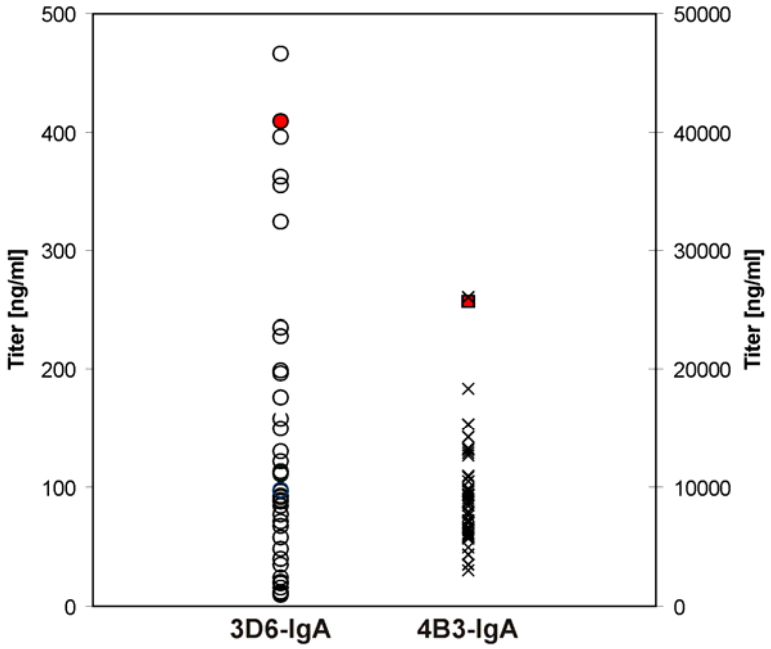

Figure 17. Product titers of the 48 best performing transfectants during the initial screening of 4B3-IgA and 3D6-IgA subclones. The four "best performing" subclones were selected for long-term comparisons for each cell line, whereas the red boxes indicate the finally selected subclones.

After two further screening rounds (data not shown) the four best producers were selected for 3D6-IgA (i.e. 3D6-IgA/3G10, 3D6-IgA/5C5, 3D6-IgA/8E10, 3D6-IgA/10D12) and 4B3-IgA (i.e. 4B3-IgA/5A6, 4B3-IgA/5A7, 4B3-IgA/6H2, 4B3-IgA/7C11) and seeded into spinner vessels to compare their growth rate, stability of product expression and viabilities in long-term culture.

Out of the 3D6-IgA expressing cell lines, subclone 3D6-IgA/5C5 was chosen for further cultivation and subcloning due to the best dimer formation (Figure 18; A and B) and the second highest specific productivity (Table 3).

4B3-IgA producing cell lines appeared to have rather similar expression profiles. All subclones predominantly secreted dimers and a smaller fraction of monomers (Figure 18;

C and D). Subclone 4B3-IgA/6H2 was selected for subcloning due to a high specific productivity along with the highest specific growth rate (Table 3).

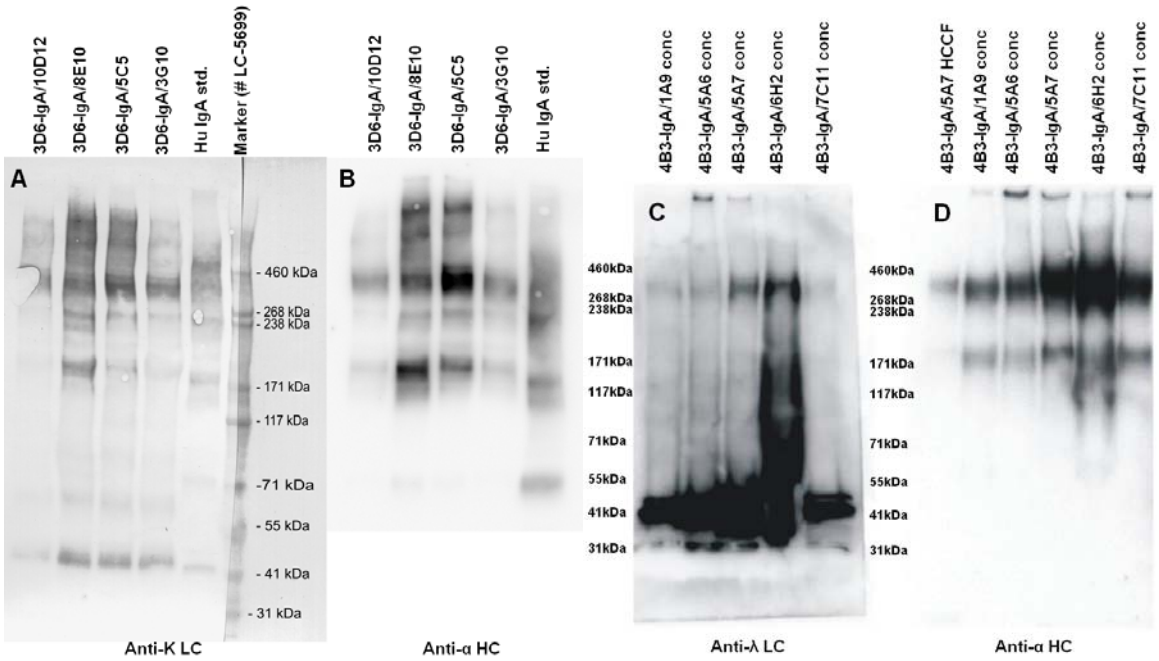

Figure 18. Western blot of the selected subclones (1st screening). Crude culture supernatant was applied for electrophoretic separation of (A and B) 3D6-IgA subclones; (C and D) 4B3-IgA subclones were concentrated 10× using Microcon YM-10 (MWCO 10 kDa) centrifugal filter units (Millipore, MA) according to the manufacturer's instructions.

The selected cell lines 3D6-IgA/5C5 and 4B3-IgA/6H2 were subcloned a second time, gene amplified with MTX (0.4 µM) and screened by ELISA for the best producers as described above. After several rounds of ELISA screening, four 3D6-IgA subclones (3D6-IgA/5C5/11E8, 3D6-IgA/5C5/13E9, 3D6-IgA/5C5/15C9, 3D6-IgA/5C5/15G6) were selected for a more detailed characterization. The selected subclones had a comparable antibody expression profile as shown in Figure 19 (A and B). Furthermore, MTX amplification increased specific productivities approximately 4-fold. Due to a high specific productivity and good growth properties subclone 3D6-IgA/5C5/13E9 was chosen for a third subcloning procedure (Table 3).

The four selected 4B3-IgA subclones (4B3-IgA/6H2/2E2, 4B3-IgA/6H2/4C11, 4B3-IgA/6H2/4G4, 4B3-IgA/6H2/5C2) looked almost identical in the Western blot (Figure 19; C and D). All subclones secreted only dimeric IgA and excessive amounts of free LC. In contrast to 3D6-IgA subclones, MTX amplification only slightly increased the 4B3-IgA antibody titers. Due to a high specific productivity subclone 4B3-IgA/6H2/4G4 was picked for a third subcloning procedure. It should be noted that other clones with higher specific productivities were discarded since their nuclear size distribution appeared tetraploid in the Coulter counter peaks (data not shown).

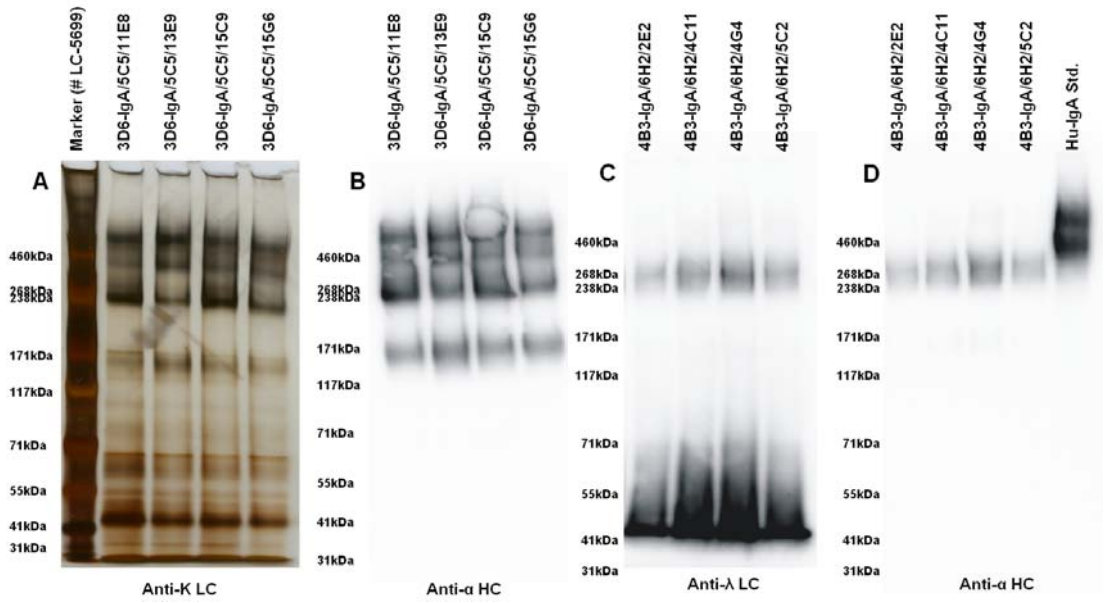

Figure 19. Western blot of selected subclones (2nd screening). Crude culture supernatant was applied for electrophoretic separation of (A and B) 3D6-IgA subclones; (C and D) 4B3-IgA subclones were concentrated 10× using Microcon YM-10 (MWCO 10 kDa) centrifugal filter units.

After the 3rd subcloning round, 3D6-IgA subclones (3D6-IgA/5C5/13E9/3A9, 3D6-IgA/5C5/13E9/3E12, 3D6-IgA/5C5/13E9/11H7, 3D6-IgA/5C5/13E9/14H3, 3D6-IgA/5C5/13E9/15B3) had a very similar expression profile of mostly dimeric and higher oligomerized IgA (Figure 20; A and B). Due to the best overall production properties in terms of specific productivity and specific growth rate (Table 3) subclone 3D6-IgA/5C5/13E9/11H7 was finally selected as the production cell line.

Similar to the 1st and 2nd subcloning round, 4B3-IgA subclones (4B3-IgA/6H2/4G4/1A4, 4B3-IgA/6H2/4G4/1G5, 4B3-IgA/6H2/4G4/5D1, 4B3-IgA/6H2/4G48B2) appeared homogeneous after the 3rd subcloning step. All subclones secreted mostly dimeric IgA in addition to a small fraction of monomeric IgA (Figure 20; C). Finally, subclone 4B3-IgA/6H2/4G4/1A4 was selected as the production cell line due to the highest specific productivity and an acceptable specific growth rate (Table 3). It should be noted that all values given in Table 3 represent mean values which were averaged over the given passage numbers.

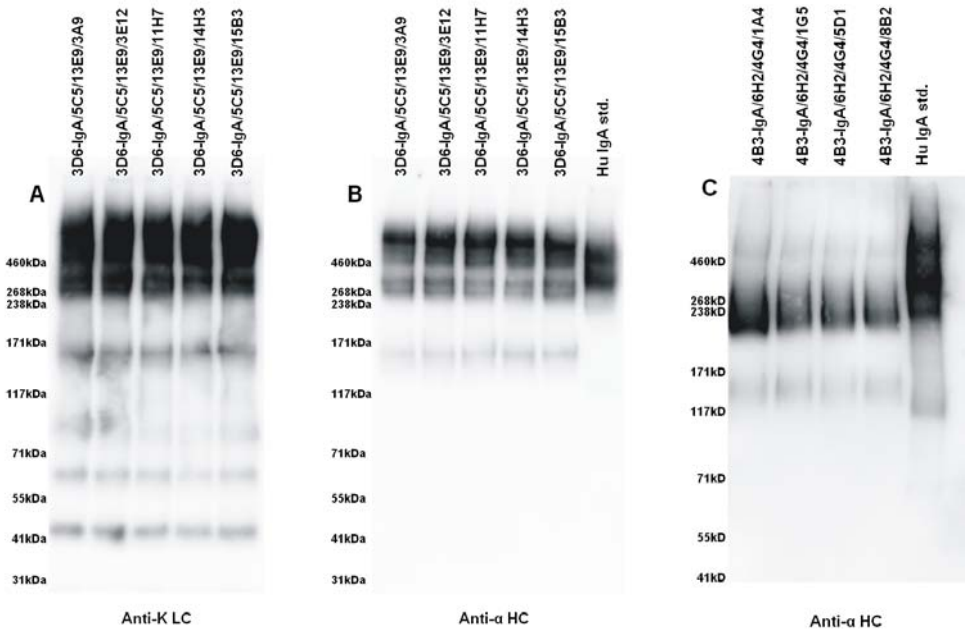

Figure 20. Western blot of selected subclones (3rd screening). Crude culture supernatant was applied for electrophoretic separation of (A and B) 3D6-IgA subclones; (C) 4B3-IgA subclones were concentrated 10× using Microcon YM-10 (MWCO 10 kDa) centrifugal filter units.

Table 3. Determined specific productivities, growth rates and product titers during routine culture of 3D6-IgA and 4B3-IgA subclones in Spinner vessels.

Subclone designation	qp [pg/cell×day]	μ [1/d]	Titer [μg/mL]	MTX [μM]	Passages [#]
3D6-IgA/3G10	2.7±0.9	0.2±0.1	9.2±3.5	0.1	37
3D6-IgA/5C5	4.5±1.8	0.3±0.1	14.7±5.4	0.1	52
3D6-IgA/8E10	6.1±2.2	0.3±0.1	18.7±6.0	0.1	32
3D6-IgA/10D12	4.0±1.3	0.2±0.1	12.8±1.3	0.1	50
3D6-IgA5C5/11E8	14.4±4.6	0.3±0.1	29.0±13.7	0.4	28
3D6-IgA5C5/13E9	16.0±6.4	0.3±0.1	34.1±16.2	0.4	28
3D6-IgA5C5/15C9	18.7±7.9	0.3±0.1	54.7±15.7	0.4	28
3D6-IgA5C5/15G6	14.7±5.1	0.3±0.1	43.0±11.6	0.4	28
3D6-IgA5C5/13E9/3A9	14.7±4.4	0.4±0.1	41.6±16.6	0.4	14
3D6-IgA5C5/13E9/3E12	13.1±3.9	0.4±0.1	30.0±9.6	0.4	13
3D6-IgA5C5/13E9/11H7	17.0±2.7	0.4±0.1	42.1±10.7	0.4	14
3D6-IgA5C5/13E9/14H3	10.5±4.0	0.3±0.1	28.9±12.2	0.4	13
3D6-IgA5C5/13E9/15B3	13.6±4.0	0.4±0.1	34.1±9.6	0.4	38
4B3-IgA/5A6	0.04±0.02	0.21±0.07	0.04±0.02	0.1	25
4B3-IgA/5A7	0.12±0.07	0.17±0.08	0.40±0.15	0.1	25
4B3-IgA/6H2	0.11±0.04	0.28±0.09	0.33±0.10	0.1	45
4B3-IgA/7C11	0.04±0.03	0.20±0.14	0.17±0.09	0.1	22
4B3-IgA6H2/2E2	0.17±0.09	0.28±0.11	0.46±0.24	0.4	29
4B3-IgA6H2/4C11	0.19±0.09	0.31±0.09	0.56±0.27	0.4	30
4B3-IgA6H2/4G4	0.13±0.07	0.31±0.10	0.58±0.40	0.4	27
4B3-IgA6H2/5C2	0.15±0.12	0.33±0.09	0.42±0.35	0.4	30
4B3-IgA6H2/4G4/1A4	0.32±0.13	0.23±0.05	0.92±0.29	0.4	26
4B3-IgA6H2/4G4/1G5	0.11±0.05	0.34±0.15	0.44±0.11	0.4	9
4B3-IgA6H2/4G4/5D1	0.13±0.03	0.39±0.04	0.37±0.13	0.4	42
4B3-IgA6H2/4G4/8B2	0.06±0.05	0.31±0.11	0.26±0.12	0.4	23

3.2 Cell line and protein characterization

3.2.1 Nucleic acid quantification

qPCR was performed in six technical replicates. The Cq values and calculated efficiencies were well reproducible as shown in Table 4. gDNA analysis revealed an overall higher exogenic GCN for the low producer 4B3-IgA than for 3D6-IgA (Figure 21). On the genomic level clone 4B3-IgA contained two times more HC, three times more JC and four times more LC than the 3D6-IgA reference clone. Both clones incorporated more HC genes than JC or LC. A possible explanation for these observations could be the presence of the dhfr amplification gene on the HC plasmid, whereas the neomycin resistance gene was located on the JC plasmid. No selection marker was included on the LC plasmid.

Despite higher gene copy numbers, 4B3-IgA exhibited only half of HC and JC transcripts compared to 3D6-IgA. LC was transcribed with the same range of efficiency and resulted in three times more LC mRNA copies. In contrast to gDNA results, LC mRNA content greatly exceeded that of HC and JC in both clones (Figure 21).

Table 4. Calculated efficiencies (E), Cq and ∆Cq values and copies relative to ß-actin for gDNA and cDNA derived from clones 3D6-IgA and 4B3-IgA.

GOI	Target	Clone	Cq	SD [%]	E	SD [%]	ΔCq ß-actin	Copies relative to ß-actin
ß-actin	gDNA	3D6-IgA	24.60	0.20	2.07	2.22	n/a	n/a
		4B3-IgA	24.21	0.14	2.07	2.22	n/a	n/a
	cDNA	3D6-IgA	18.52	0.13	2.03	0.43	n/a	n/a
		4B3-IgA	16.25	0.63	2.04	1.33	n/a	n/a
HC	gDNA	3D6-IgA	23.56	0.16	1.95	3.32	-1.03	8.28
		4B3-IgA	22.11	0.14	1.95	3.32	-2.11	16.44
	cDNA	3D6-IgA	21.78	0.17	1.91	1.35	3.26	0.38
		4B3-IgA	19.50	0.68	1.97	1.53	3.25	0.20
JC	gDNA	3D6-IgA	24.81	0.03	1.95	0.94	0.22	3.80
		4B3-IgA	22.77	0.10	1.95	0.94	-1.44	11.20
	cDNA	3D6-IgA	24.52	0.23	1.82	0.87	5.97	0.22
		4B3-IgA	20.81	1.54	1.96	0.27	4.56	0.10
LC	gDNA	3D6-IgA	24.90	0.14	2.05	0.59	0.31	0.98
		4B3-IgA	21.50	0.21	2.11	1.21	-2.71	4.40
	cDNA	3D6-IgA	20.26	0.20	1.88	0.75	1.73	1.30
		4B3-IgA	15.02	2.36	1.98	1.30	-1.22	3.93

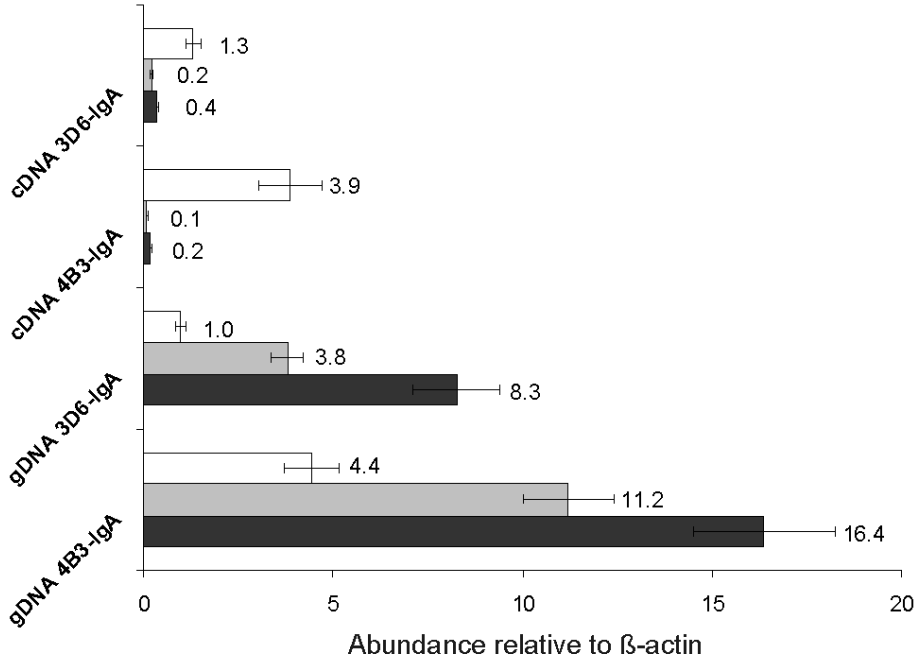

Figure 21. Gene copy number and transcript level of recombinant clones expressing 3D6-IgA or 4B3-IgA. The abundance of LC (□), JC (▒) and HC (■) genes was calculated relative to ß-actin.

3.2.2 Flow cytometry

Intracellular product content and potential ER stress signals were investigated by flow cytometry of recombinant and host cell lines. As shown in Figure 22, all examined parameters were homogeneously allocated in each cell population. The intracellular LC signal of clone 4B3-IgA, relative to the λ-stained negative control, was lower than that of clone 3D6-IgA, relative to the κ-stained host cell line. HC polypeptides were slightly more abundant in the low producer 4B3-IgA.

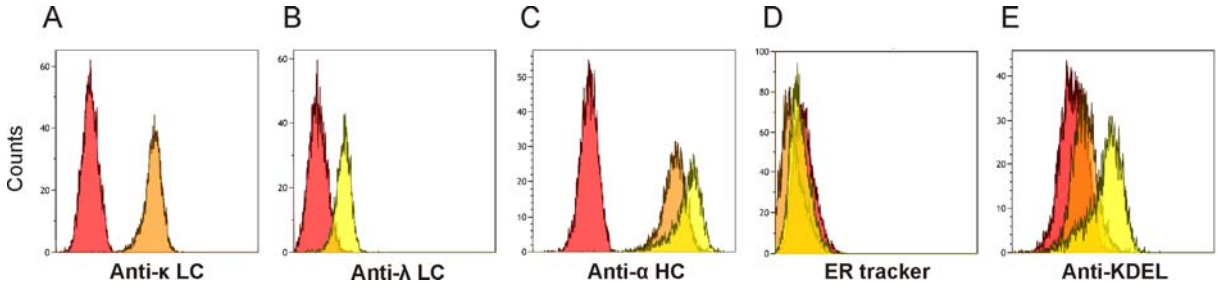

Figure 22. Cell characterization by flow cytometry. The histograms show the recombinant clones 3D6-IgA (▒) and 4B3-IgA (□) as well as CHO host cell line (■) after staining with (A) anti-κ LC, (B) anti-λ LC, (C) anti-α HC, (D) ER tracker Blue/White DPX and (E) anti-KDEL reagents.

Furthermore, live-cell ER staining disclosed similarly intense signals for recombinant and host cell lines, which indicated that their ERs were of comparable sizes. Noteworthy differences were found with an antibody that is specific to the KDEL sequence present on several ER-retained chaperones such as BiP, PDI, GRP94 (Xiao et al., 1999) and CRT (Weclewicz et al., 1998). The signal was stronger for the low producer (4B3-IgA) than for

the reference clone 3D6-IgA than for the host cell line and adumbrated an increasing ER stress.

3.2.3 Immunofluorescence microscopy

Immunofluorescence microscopy confirmed results obtained from flow cytometry. DAPI staining of the nucleus and live-cell staining of the ER appeared similar in size, distribution and structure among the recombinant and host cell lines (data not shown). The LC signal of clone 4B3-IgA was well-distributed throughout the cell and appeared rather faint. The HC seemed spread equally within the cell interior but several large, granule-like HC spots could also be visualized. In the overlay of LC and HC only a few spots appeared in white, indicating co-localized polypeptides (Figure 23). When we looked at the reference clone 3D6-IgA the LC/HC distribution was similar but the granule-like HC structures were absent. In order to investigate the assembly status of the antibody polypeptide chains, the intra- and extracellular product was immunoblotted in the following experiment.

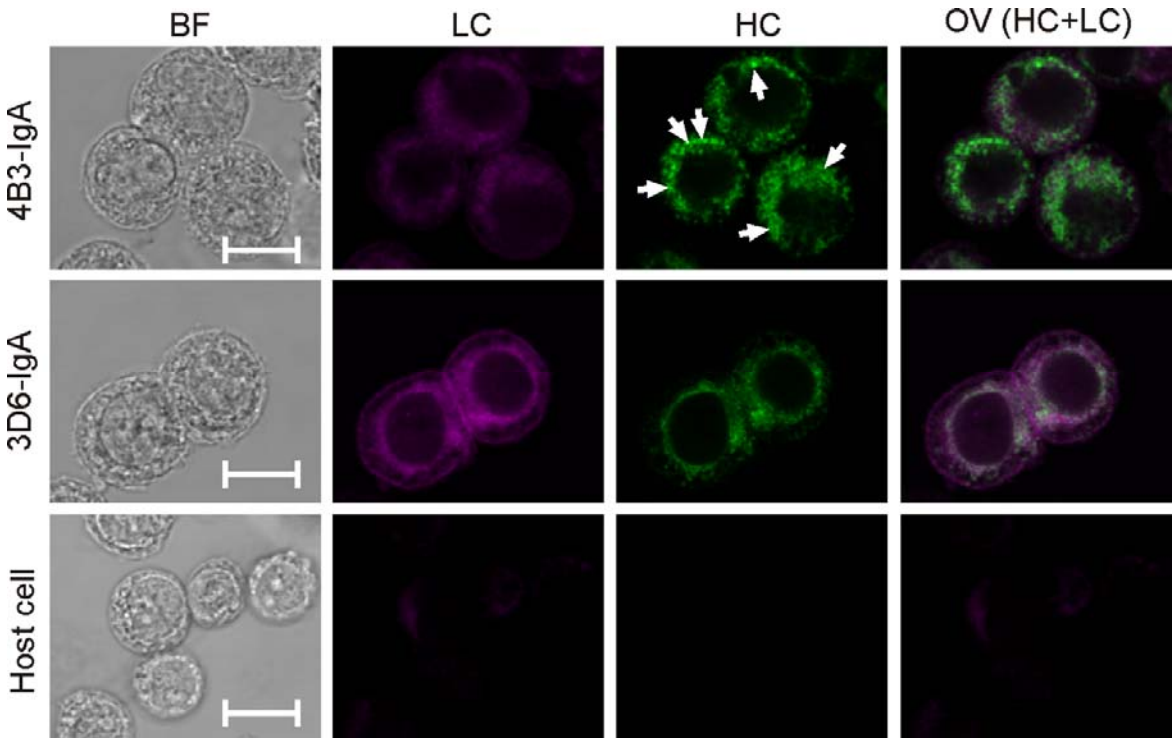

Figure 23. Immunofluorescence microscopy of cell lines. The recombinant clones 3D6-IgA and 4B3-IgA as well as CHO host cell line were bright field (BF) imaged. Intracellular LC and HC was detected and stained in purple and green, respectively. Co-localized LC and HC are shown in white color in the overlay (OV). Bar, 10 µm.

3.2.4 Immunoblotting of intracellular and secreted product

In the previously performed Western blots (Figure 18 to Figure 20) 3D6-IgA and 4B3-IgA culture supernatants visualized diverse IgA isoforms. Both IgAs were secreted as

monomers and dimers, however, with different predominance. Only the high-producing 3D6-IgA subclones secreted higher polymerized antibody isoforms. Even more tri- and tetrameric IgA was formed when the subclones were gene amplified. In general, high molecular weight IgAs with 20-80% dimeric IgA were similarly observed by other groups in hybridomas as well as recombinant CHO cells (Berdoz et al., 1999). Also tetramers, pentamers as well as hexamers were reported (Sørensen et al., 1999; Johansen et al., 2001; Favre et al., 2003).

Anti-α chain staining of clone 4B3-IgA's intracellular content emerged as a broad smear distributed among several different molecular weights but most intensely above the position of monomeric IgA (Figure 24; A). The respective band had a heterogeneous constitution since HC, LC and JC polypeptides were determined. Staining of the culture supernatant illustrated that only dimers were secreted, while the majority of the HC fraction remained secretion incompetent. This discovery indicated incorrectly folded and/or misassembled polypeptides which were intracellularly retained and hence accumulated in subclone 4B3-IgA. These data matched well with observations from immunofluorescence microscopy in which polypeptide aggregation was similarly seen (Figure 23).

The cellular lysate of subclone 3D6-IgA contained only sharp bands of IgA mono- and dimers. Also tri- and tetrameric IgA was found but only in the culture supernatant (Figure 24; B). This was an interesting finding since it indicated that IgA oligomerization started after product secretion. No intracellular bands suggested protein aggregation or assembly with chaperones as observed for subclone 4B3-IgA.

Intra- and extracellular fractions of the low producer subclone 4B3-IgA comprised an excess of unbound LC. Only small amounts of free LC were visualized in subclone 3D6-IgA's cell lysate as well as in the supernatant. Free HC was not found in any fraction. As expected, all oligomeric molecules contained JC polypeptides in contrast to monomeric IgAs.

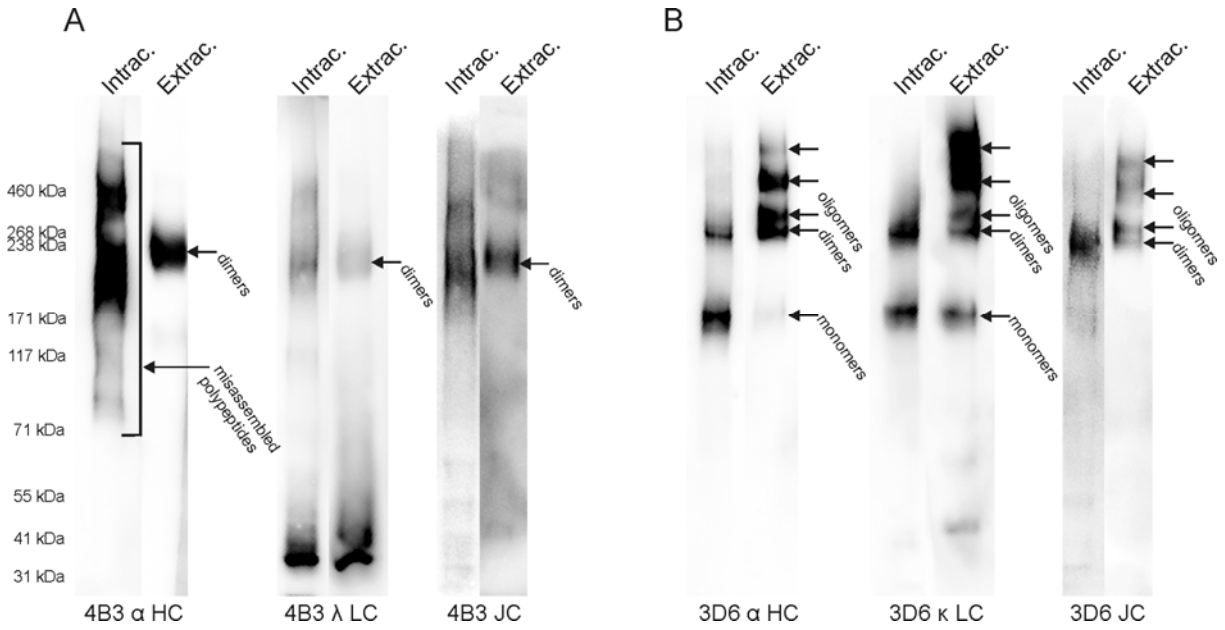

Figure 24. Immunoblot of intracellularly expressed polypeptides and secreted product. Protein isolates from the recombinant cell lines (A) 3D6-IgA and (B) 4B3-IgA were stained individually for their HC, LC and JC molecules.

3.3 Recombinant expression of secretory IgA

All screened 3D6-sIgA and 4B3-sIgA transfectants had very low antibody titers which were mostly in the range of the blank of the sIgA ELISA itself. The wells with the highest signals were selected (data not shown) as described previously. Finally, five subclones were chosen for long-term characterization. The selected 3D6-sIgA subclones secreted antibodies at rather low concentrations (Table 5). Western blots visualized that all subclones (except 3D6-sIgA/2E8) predominantly produced monomeric IgA and only a small fraction of secretory IgA (Figure 25).

Antibody expression of all 4B3-sIgA subclones was very poor and often below the ELISA detection range (Table 5). No product could be detected during Western blotting, even after concentration of culture supernatants (Figure 25). As a consequence for the low sIgA titers, an alternative approach was developed in which human secretory component was mixed with dimeric IgA to produce secretory IgA (see section 3.5). It should be noted that all values given in Table 5 represent mean values which were averaged over the given passage numbers.

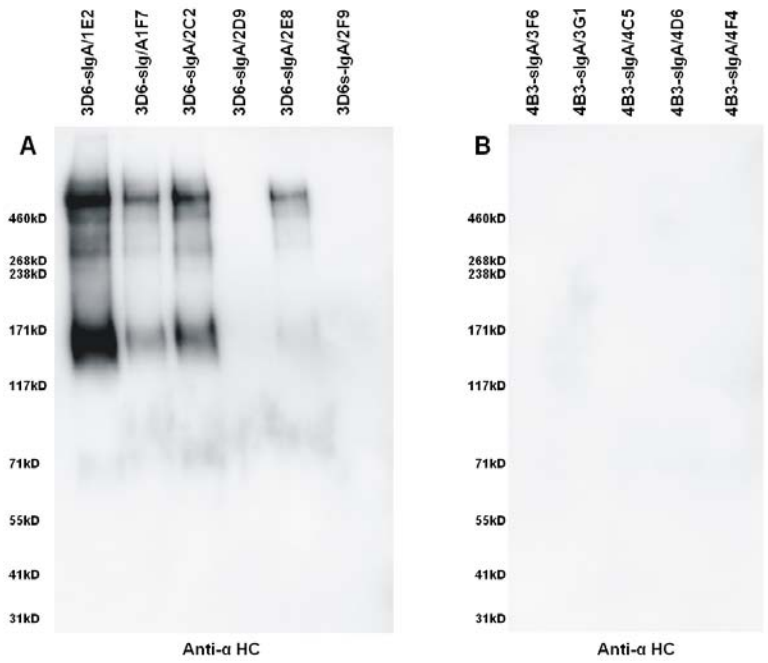

Figure 25. Western blot of (A) 3D6-sIgA and (B) 4B3-sIgA selected subclones (1st subclones). Culture supernatants were concentrated 20× using Microcon YM-10 (MWCO 10 kDa) centrifugal filter units prior to electrophoretic separation. Samples were detected via an HRP-conjugated anti-α antiserum.

Table 5. Determined specific productivities, growth rates and product titers during routine culture of 3D6-sIgA and 4B3-sIgA clones and subclones in Spinner vessels.

Subclone designation	qp [pg/cell×day]	μ [1/d]	Titer [μg/mL]	MTX [μM]	Passages [#]
3D6sIgA1E2	1.89±0.91	0.18±0.07	3.62±0.68	0.1	23
3D6sIgA1F7	0.90±0.40	0.24±0.07	2.11±0.76	0.1	31
3D6sIgA2C2	0.91±0.33	0.31±0.07	2.34±0.85	0.1	22
3D6sIgA2D9	0.17±0.09	0.27±0.09	0.31±0.25	0.1	20
3D6sIgA2E8	0.31±0.15	0.29±0.08	0.79±0.23	0.1	20
4B3sIgA3F6	Na	0.22±0.07	0.36±0.24	0.1	23
4B3sIgA3G1	0.39±0.22	0.22±0.13	0.85±0.45	0.1	31
4B3sIgA4C5	Na	0.28±0.07	0.33±0.29	0.1	22
4B3sIgA4D6	0.34±0.21	0.32±0.08	1.02±0.38	0.1	20
4B3sIgA4F4	0.56±0.19	0.35±0.14	1.14±0.72	0.1	20

3.4 Recombinant expression of human secretory component

After transfection the 12 subclones with the highest titers were analyzed by Western blotting to investigate the quality of the secreted hSC product. As expected, the band for hSC was approximately 80 kDa (Lüllau et al., 1996; Berdoz et al., 1999) and appeared at the same height for all analyzed clones (Figure 26). Subclone hSC-5G7 was chosen as the production cell line due to the highest specific productivity and a high specific growth rate as shown in Table 6. It should be noted that all values given in Table 6 represent mean values which were averaged over the given passage numbers.

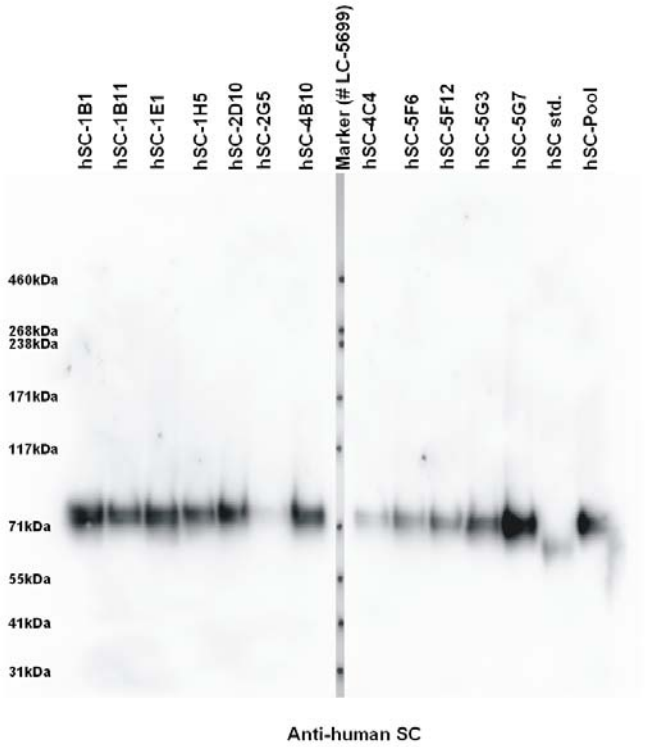

Figure 26. Western blot of hSC selected subclones. Crude culture supernatants were applied onto the gel. Standard was recombinant human SC. Samples were detected via a mouse anti-human SC antiserum and a HRP-conjugated anti-mouse-γ-chain antiserum.

Table 6. Determined specific productivities, growth rates and product titers during routine culture of hSC clones in Spinner vessels.

Subclone designation	qp [pg/cell×day]	μ [1/d]	Titer [μg/mL]	MTX [μM]	Passages [#]
hSC-5G7	17.21±5.24	0.37±0.08	37.48±14.13	0.1	37
hSC-4B10	6.20±2.12	0.43±0.07	16.74±5.24	0.1	22
hSC-2D10	9.17±3.41	0.29±0.10	20.56±9.06	0.1	21
hSC-1E1	1.96±1.36	0.38±0.05	6.34±3.72	0.1	18

3.5 Assembly of secretory IgA by in vitro association of hSC with IgA

The production of sIgA from mixing polymeric IgA with hSC was investigated at three different molar ratios and two temperatures for both 3D6-IgA and 4B3-IgA. As shown in Table 7 a molar ratio of 1:1 yielded the lowest sIgA concentrations. Increasing the hSC concentration resulted in higher sIgA formation. Moreover, higher sIgA yields could be achieved when the reaction mixture was incubated at 37°C as compared to room temperature. A major difference between the two IgA antibodies was that sIgA production was in any case more efficient for 4B3-IgA (max. 99.9% sIgA) than for 3D6-IgA (max. 39.6% sIgA).

Figure 27 shows a Western blot of the different sIgA reaction mixtures incubated at 37°C only. It is obvious that all three tested molar ratios still contained free hSC polypeptides,

however, this could result from a non-covalent association with polymeric IgA which is not stable during denaturing electrophoresis conditions. The other fraction of hSC is covalently associated with polymeric IgA. Another noteworthy result is that hSC associated with polymeric IgA alone while IgA monomers did not contain any hSC.

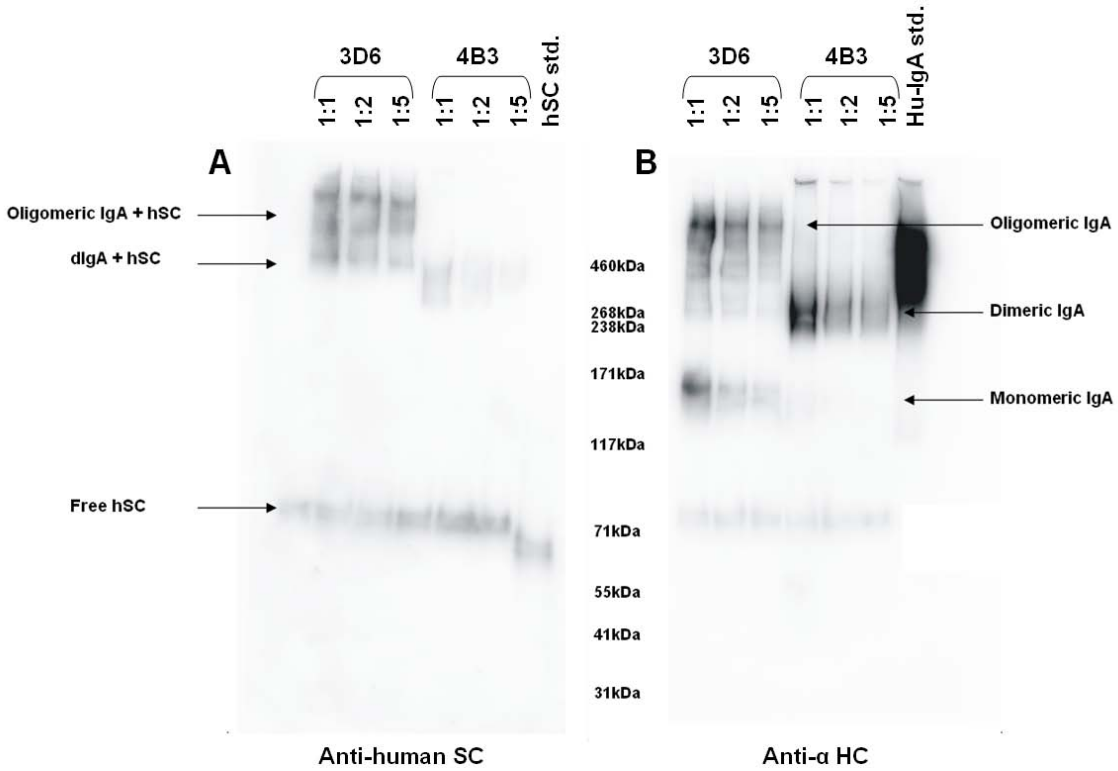

Figure 27. Western blot of 3D6-sIgA and 4B3-sIgA produced by association of hSC with IgA. Only samples which were incubated at 37°C are shown. The tested molar ratios of IgA:hSC are indicated above the sample lane. (A) Samples were detected via a mouse anti-human SC antiserum and a HRP-conjugated anti-mouse-γ-chain antiserum; (B) via a HRP-conjugated anti-α chain antiserum.

Table 7. Samples were mixed and subsequently incubated at 22°C and 37°C, 300rpm for 6 hours. Quantification was then performed by ELISA.

	Temperature [°C]	Molar ratio dIgA:hSC	dIgA [µg]	hSC [µg]	dIgA [µg/ml]	sIgA [µg/ml]	Yield [% sIgA]
3D6	22	1:1	8	1.8	72.7	18.1	24.9
		1:2	8	3.6	72.7	18.2	25.0
		1:5	8	9.0	72.7	23.2	31.9
	37	1:1	8	1.8	72.7	23.7	32.6
		1:2	8	3.6	72.7	27.6	37.9
		1:5	8	9.0	72.7	28.8	39.6
4B3	22	1:1	8	1.8	72.7	48.6	66.9
		1:2	8	3.6	72.7	53.8	74.0
		1:5	8	9.0	72.7	65.7	90.3
	37	1:1	8	1.8	72.7	60.8	83.6
		1:2	8	3.6	72.7	59.6	82.0
		1:5	8	9.0	72.7	72.6	99.9

3.6 Protein purification

IgA purification was investigated by two different approaches. The first approach was termed “Multiple step chromatography” and comprised several chromatographic steps. The second approach consisted of a single step using an IgA affinity resin and was termed “Single step chromatography”.

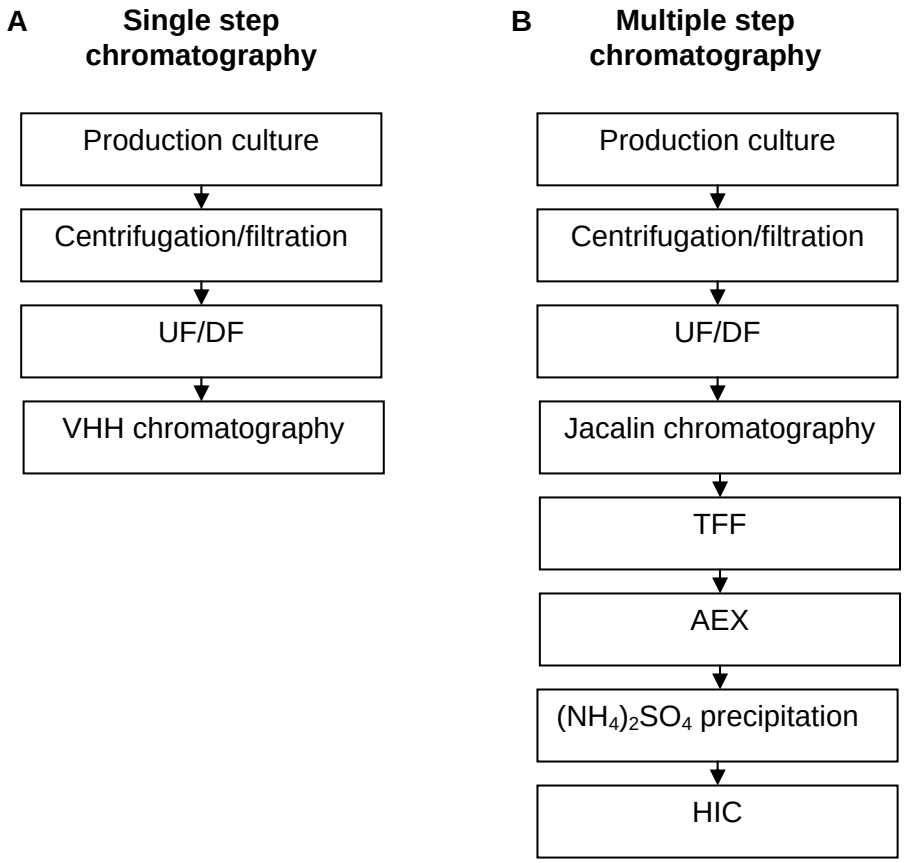

Figure 28. A schematic illustration of the two different IgA purification approaches termed (A) single step chromatography and (B) multiple step chromatography.

3.6.1 Multiple step chromatography

3.6.1.1 Ultrafiltration/diafiltration

Ultrafiltration/diafiltration (UF/DF) was performed to concentrate and reduce protein impurities and buffer exchange the culture supernatant for the subsequent chromatographic purification steps. 3D6-IgA was concentrated approximately 10× and 4B3-IgA ×40, whereas the process applied and antibody yields obtained were similar for both antibodies. As shown in Figure 30 and Figure 32 the culture supernatant was efficiently concentrated without any major product losses of a maximum of 5% (Reinhart et al., 2012). Results of a typical UF/DF procedure are shown in Table 8.

Table 8. Typical results of an UF/DF run.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	1730.00	22.83	39495.47	-
Filtrate	2450.00	0.01	21.29	0.05
Retentate	301.00	129.78	39062.28	98.90
				Total 98.95

3.6.1.2 Immobilized jacalin chromatography

Antibody purification by immobilized jacalin chromatography removed substantial amounts of impurities (especially low molecular weight proteins) from 3D6-IgA and 4B3-IgA UF/DF retentate as visualized by SDS-PAGE in the flowthrough fractions. Typically the antibody yields were around 97% for 3D6-IgA and 98% for 4B3-IgA as previously published by Reinhart et al. (2012).

Figure 29 and Figure 31 show typical chromatograms for 3D6-IgA and 4B3-IgA purification from UF/DF retentate, respectively. The fractions obtained were analyzed by silver staining of SDS-PAGE as well as by Western blotting to evaluate fraction purity and to confirm IgA identity, quality and size (Figure 30 and Figure 32). ELISA was performed to determine antibody titer and recovery yield during the individual chromatographic steps (Table 9 and Table 10).

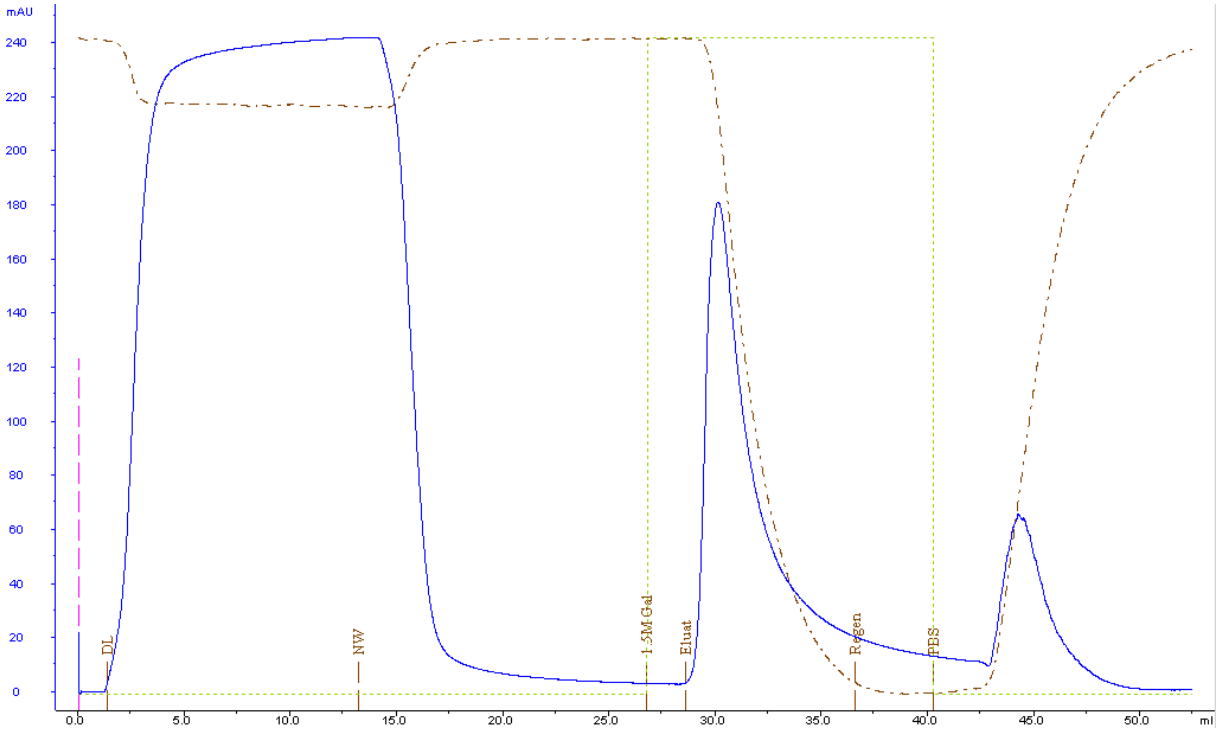

Figure 29. Typical chromatogram of 3D6-IgA purification by immobilized jacalin chromatography.

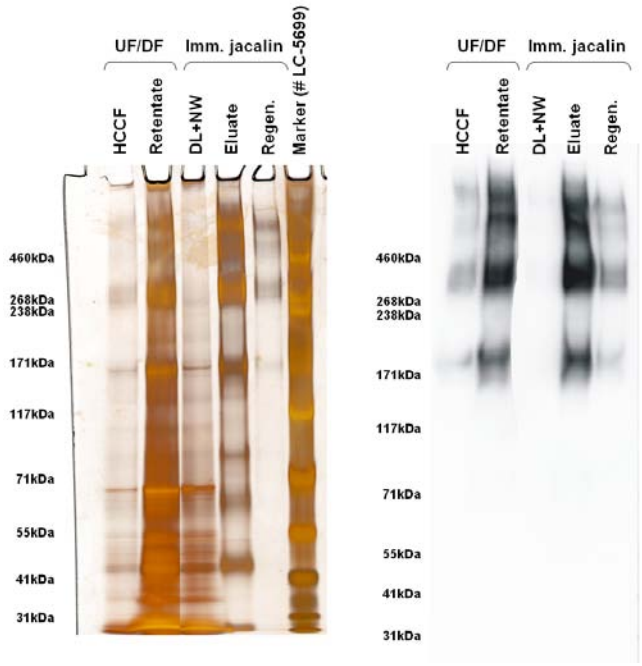

Figure 30. Typical results of an SDS-PAGE silver stain and Western blot with 3D6-IgA fractions obtained from immobilized jacalin chromatography. The Western blot was detected by anti-α chain staining. It should be noted that DL and NW refer to the "flowthrough" and "wash" fractions, respectively.

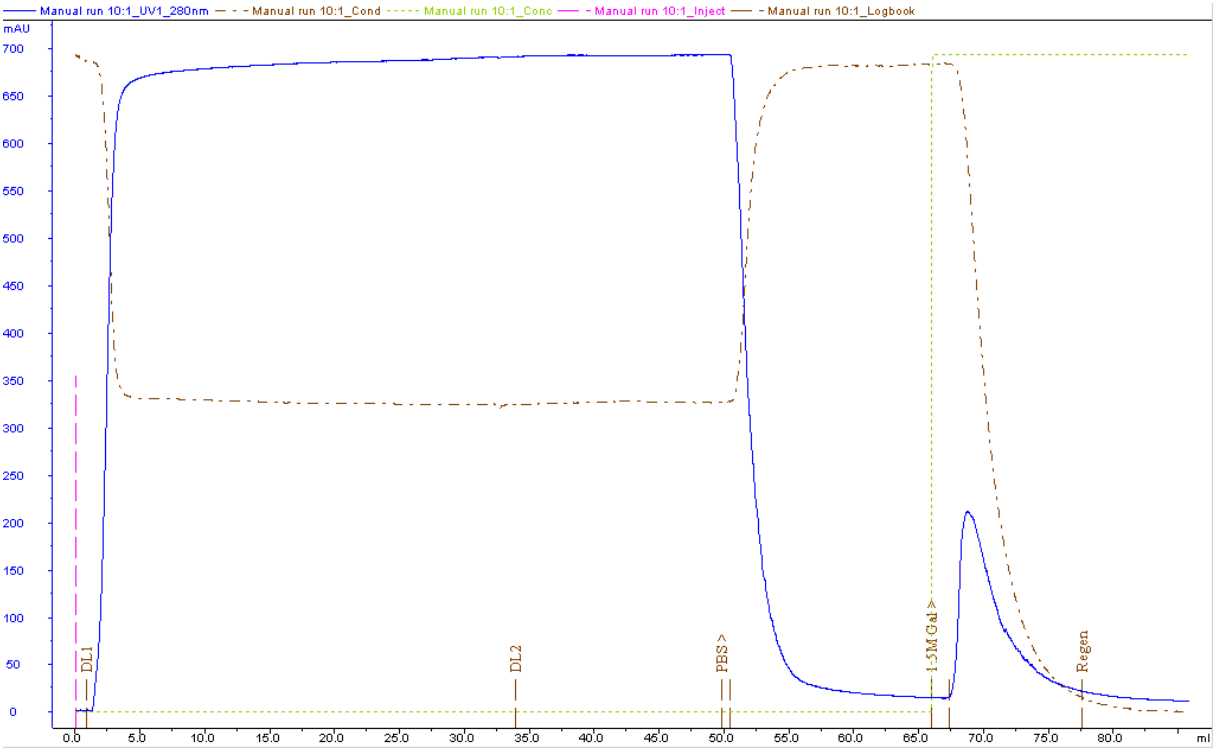

Figure 31. Typical chromatogram of 4B3-IgA purification by immobilized jacalin chromatography.

Table 9. Typical results of a chromatographic run with 3D6-IgA on immobilized jacalin.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	13.26	517.47	6861.64	-
Flowthrough /Wash	27.46	6.65	182.54	2.66
Elution	8.50	796.81	6772.91	98.71
Regeneration	17.16	0.27	4.71	0.07
				Total 101.44

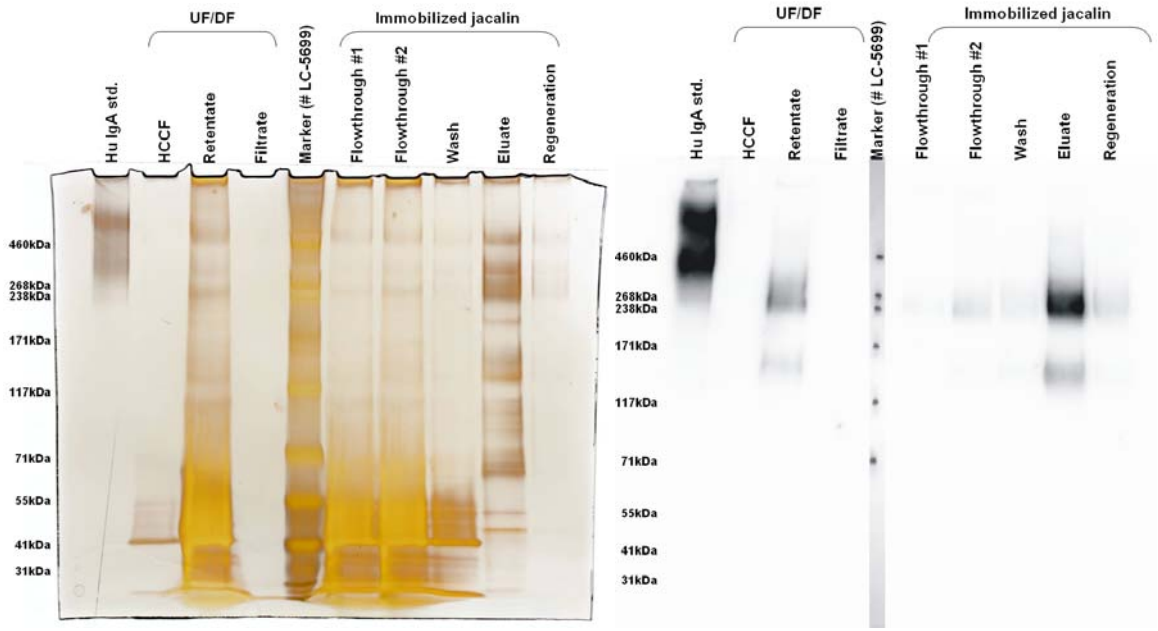

Figure 32. Typical results of an SDS-PAGE silver stain and Western blot with 4B3-IgA fractions obtained from immobilized jacalin chromatography. The Western blot was detected by anti-α chain staining.

Table 10. Typical results of a chromatographic run with 4B3-IgA on immobilized jacalin.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	50.61	57.32	2901.13	-
Flowthrough #1	33.54	3.58	119.91	4.13
Flowthrough #2	16.84	15.30	257.62	8.88
Wash	16.98	14.86	252.39	8.70
Elution	10.99	203.49	2236.31	77.08
Regeneration	9.45	23.98	226.65	7.81
				Total 106.61

3.6.1.3 Anion exchange chromatography

Anion exchange chromatography enabled a further improvement of IgA fraction purity. As described in Reinhart et al. (2012) the recovery yields of 3D6-IgA and 4B3-IgA were acceptable and roughly 87%.

Figure 33 and Figure 35 show typical chromatograms of 3D6-IgA and 4B3-IgA purification, respectively. The fractions obtained were analyzed by silver staining of SDS-PAGE as well as by Western blotting to evaluate fraction purity and to confirm IgA

identity, quality and size (Figure 34 and Figure 36). ELISA was performed to determine antibody titer and recovery yield during the individual chromatographic steps (Table 11 and Table 12).

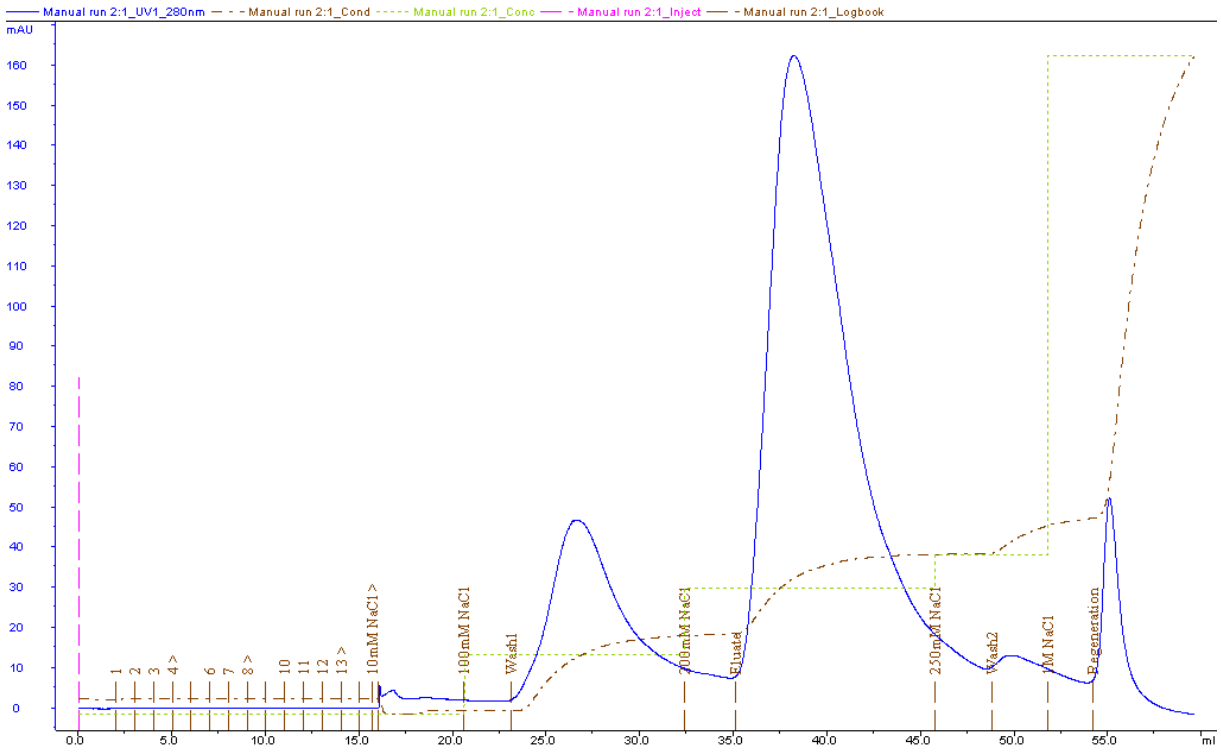

Figure 33. Typical chromatogram of 3D6-IgA purification by anion exchange chromatography.

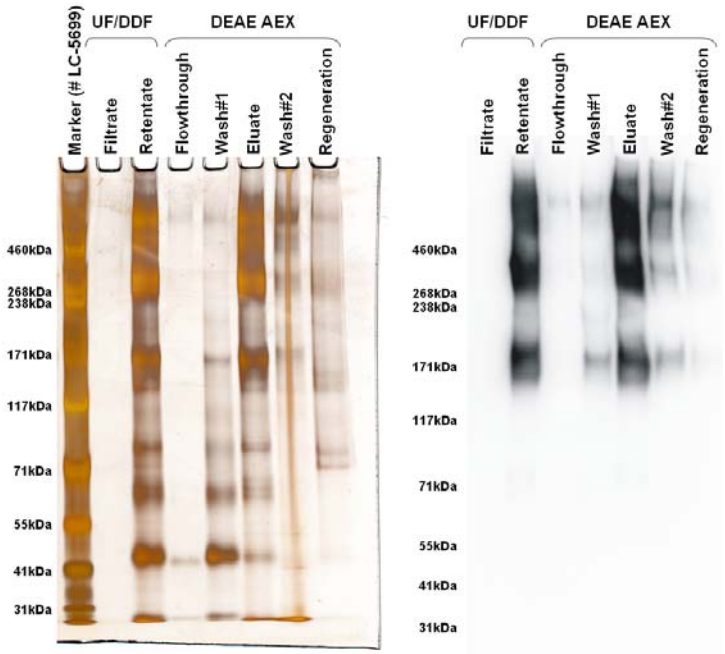

Figure 34. Typical results of an SDS-PAGE silver stain and Western blot with 3D6-IgA fractions obtained from anion exchange chromatography. The Western blot was detected by anti-α chain staining.

Table 11. Typical results of a chromatographic run with 3D6-IgA employing anion exchange chromatography.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	16.00	1405.19	22483.00	-
Flowthrough	7.07	6.76	47.78	0.8%
Wash #1	12.08	48.65	587.73	2.61
Eluation	13.90	1429.50	19870.05	88.38
Wash #2	5.46	154.34	842.70	3.75
Regeneration	5.66	54.47	308.27	1.37
				Total 96.52

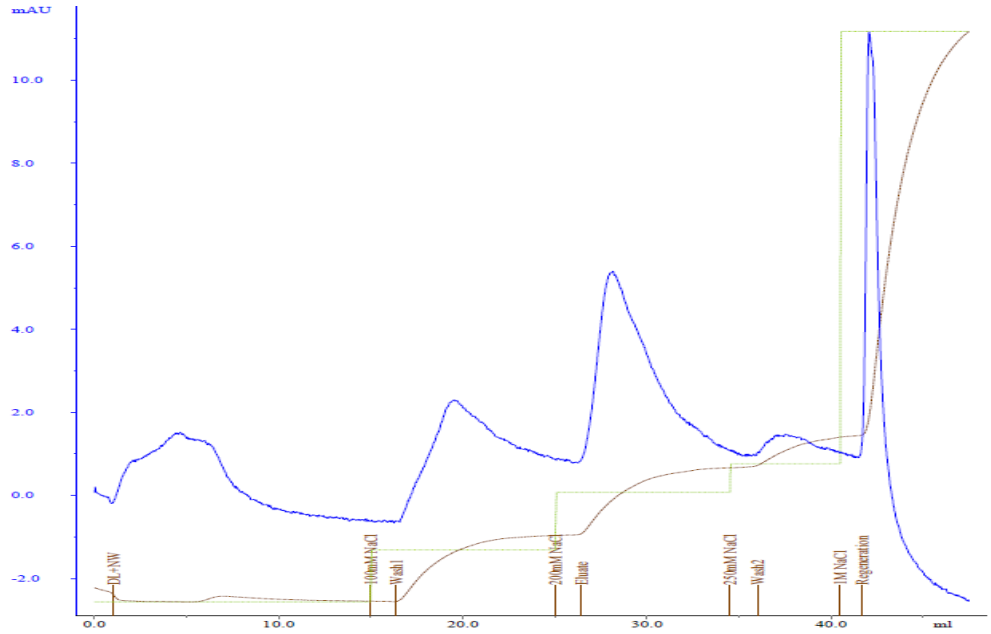

Figure 35. Typical chromatogram of 4B3-IgA purification by anion exchange chromatography.

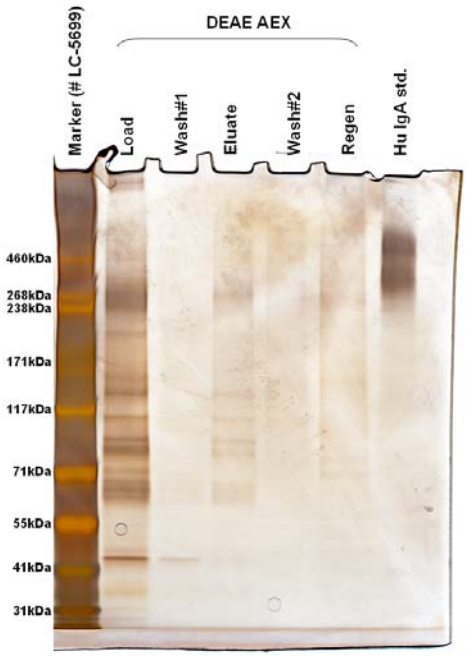

Figure 36. Typical results of an SDS-PAGE silver stain with 4B3-IgA fractions obtained from anion exchange chromatography.

Table 12. Typical results of a chromatographic run with 4B3-IgA employing anion exchange chromatography.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	13.21	38.73	511.57	-
Flowthrough	26.42	0.01	0.06	0.01
Wash #1	17.47	0.31	5.35	1.05
Eluation	26.63	35.68	443.68	86.73
Wash#2	16.15	2.63	21.63	4.23
Regeneration	7.21	2.30	16.58	3.24
				Total 95.26

3.6.1.4 Ammonium sulfate precipitation

Prior to HIC, the maximum molarity of $(NH_4)_2SO_4$ in the load before IgA precipitation was investigated. Therefore, buffer (3.6M $(NH_4)_2SO_4$, 20mM Tris, pH 7.5) was added to 250µL sample aliquots of AEX eluate (in 200mM NaCl, 20mM Tris, pH 8.5) to 0.5 M – 2 M $(NH_4)_2SO_4$ in 0.25 M steps. Samples were incubated at room temperature for 10 minutes and subsequently centrifuged at 10,000×g for 10 minutes. Supernatant was collected and vials which contained pellets (precipitated protein) were re-suspended in the original buffer volume (10mM NaCl, 20mM Tris, pH 7.5). Fractions were then separated by SDS-PAGE and analyzed by Western blotting, silver staining and ELISA. Most dimeric IgA product starts to precipitate when 1.25 M $(NH_4)_2SO_4$ is exceeded (Figure 37).

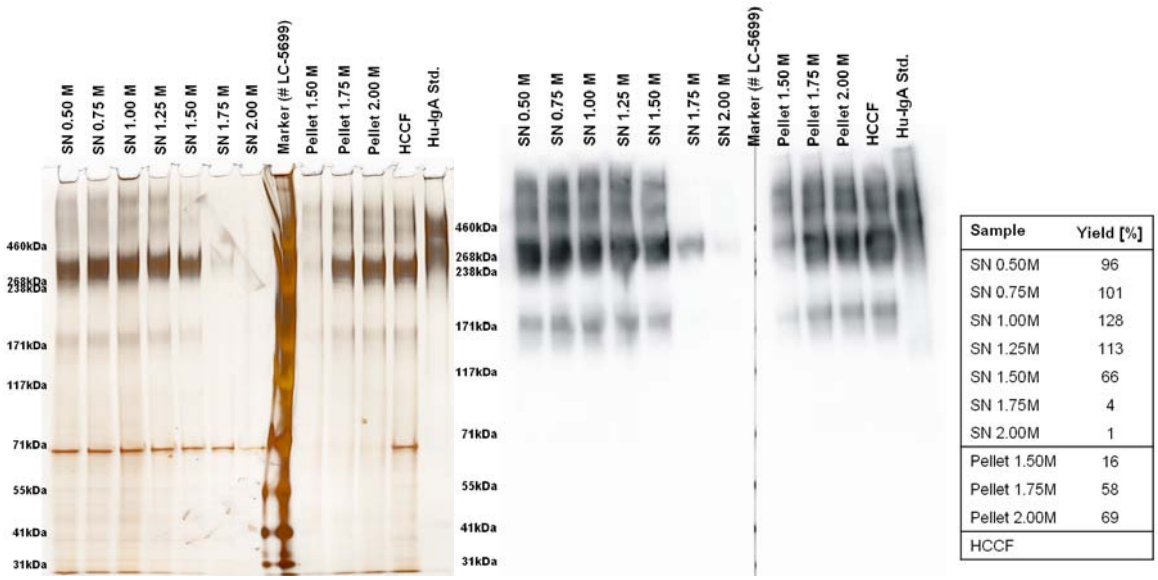

Figure 37. Silver stained SDS-PAGE and Western blot of fractions collected during 3D6-IgA precipitation with $(NH_4)_2SO_4$. Western blot was detected with a HRP-conjugated anti-α antiserum. The Western blot was detected by anti-α chain staining.

3.6.1.5 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was employed as a final purification step. HIC further reduced impurities from 3D6-IgA or 4B3-IgA eluates. Yields typically ranged between 77% (3D6-IgA) and 68% (4B3-IgA) as previously published in Reinhart et al.

(2012). Furthermore, the HIC step enabled a partial 3D6-IgA separation of dimeric IgA from higher polymerized IgA (Figure 39).

Figure 39 and Figure 41 show typical chromatograms of 3D6-IgA and 4B3-IgA purification, respectively. The fractions obtained were analyzed by silver staining of SDS-PAGE as well as Western blotting to evaluate fraction purity and to confirm IgA identity and size (Figure 39 and Figure 41). ELISA was performed to determine antibody titer and recovery yield during the individual chromatographic steps (Table 13 and Table 14).

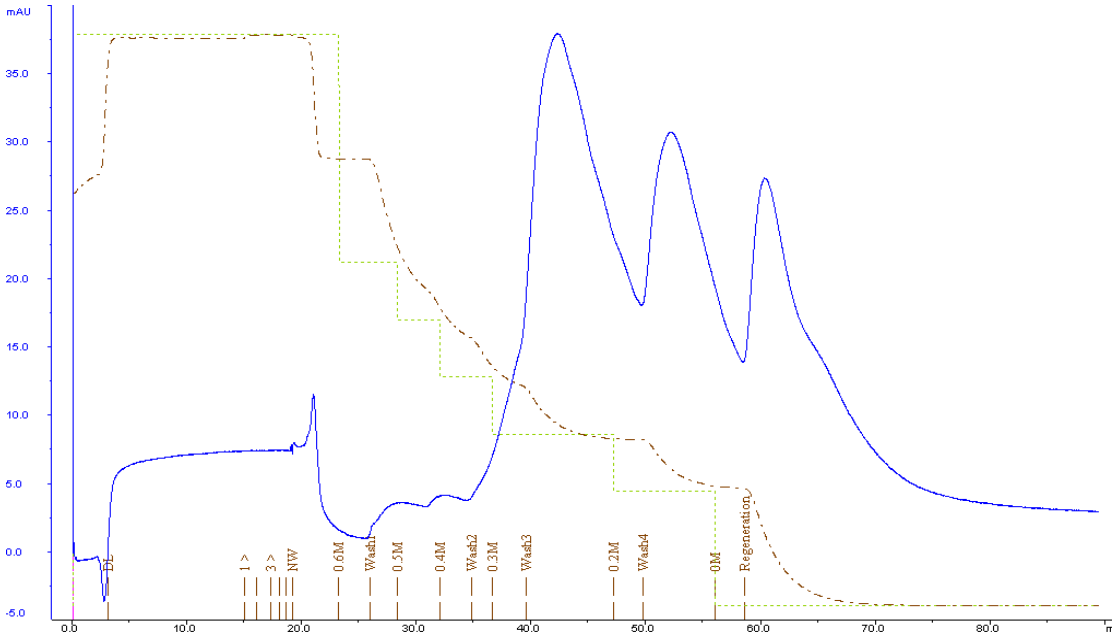

Figure 38. Typical chromatogram of 3D6-IgA purification by hydrophobic interaction chromatography.

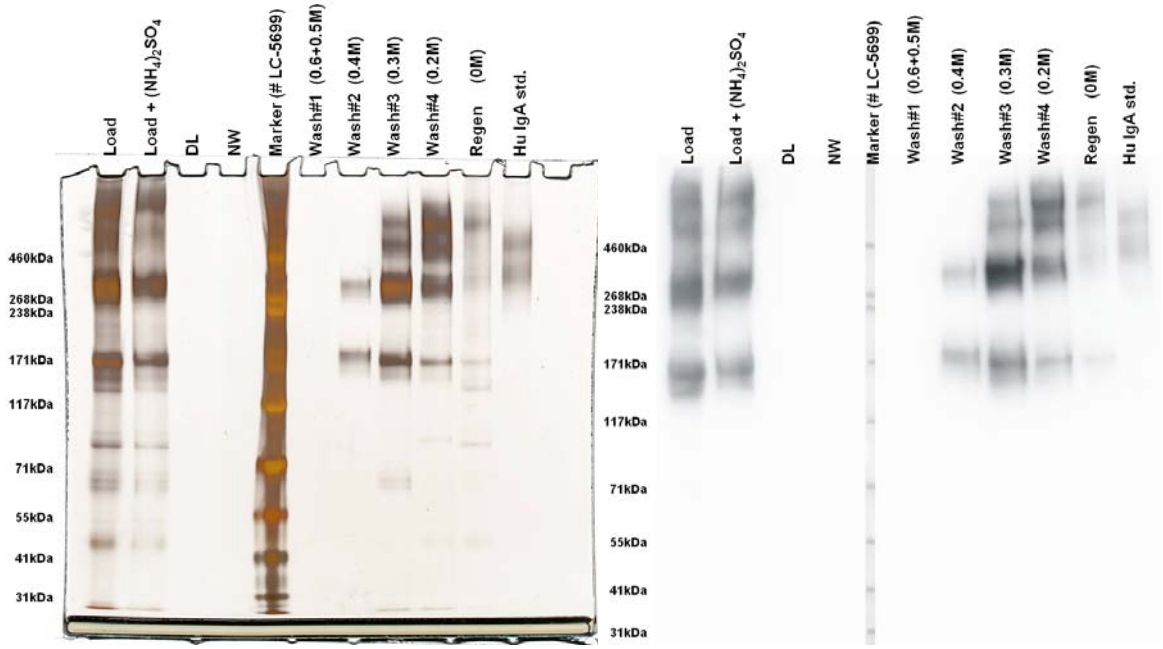

Figure 39. Typical results of an SDS-PAGE silver stain and Western blot with 3D6-IgA fractions obtained from hydrophobic interaction chromatography. The Western blot was detected by anti-α chain staining. It should be noted that DL and NW refer to the "flowthrough" and "wash" fractions, respectively.

Table 13. Typical results of a chromatographic run with 4B3-IgA employing hydrophobic interaction chromatography.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	20.61	0.01	0.20	-
Flowthrough	24.41	0.06	0.25	0.00
Wash #1	9.38	0.01	0.09	0.00
Wash #2	5.00	52.195	260.98	2.08
Wash #3	10.48	526.55	5518.24	44.07
Wash #4	9.03	372.971	3367.93	26.90
Regeneration	30.96	66.445	2057.14	16.43
				Total 89.48

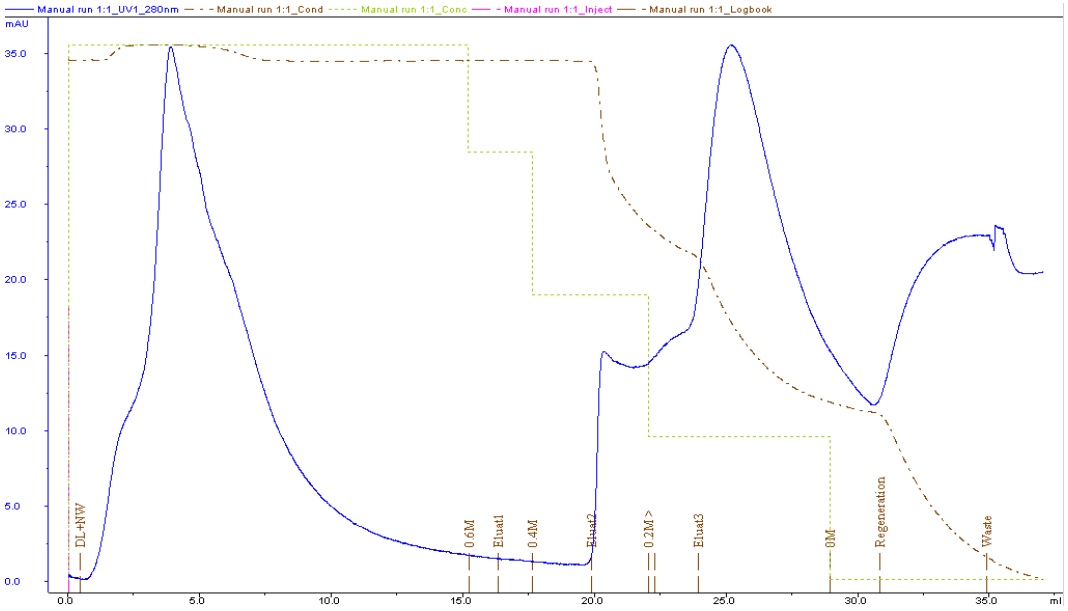

Figure 40. Typical chromatogram of 4B3-IgA purification by hydrophobic interaction chromatography.

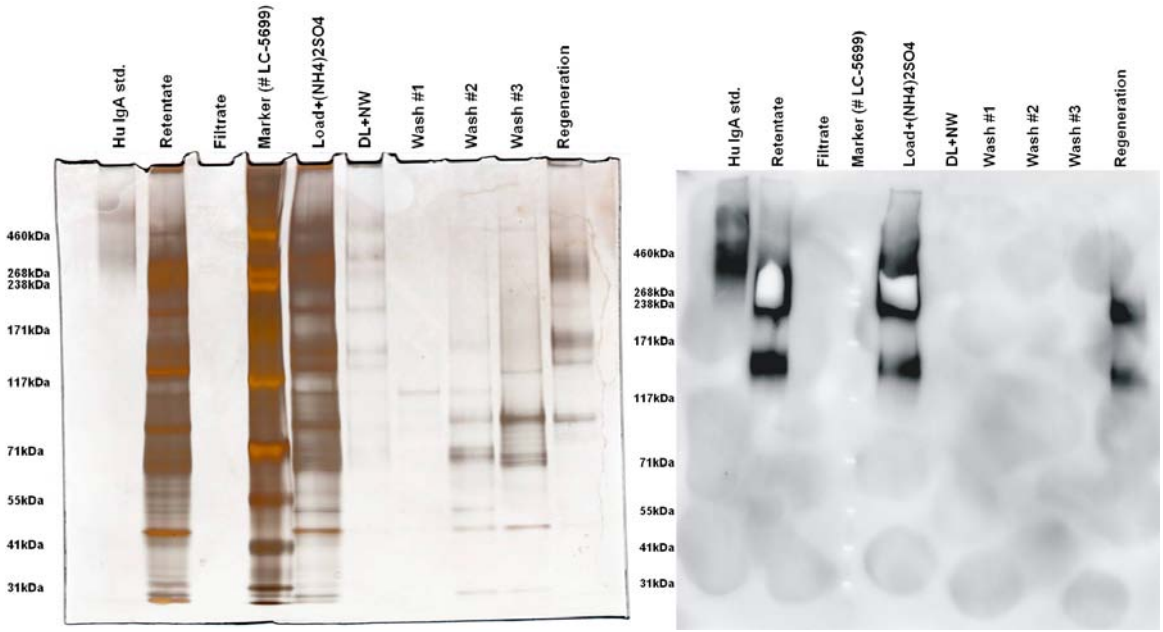

Figure 41. Typical results of an SDS-PAGE silver stain and Western blot with 4B3-IgA fractions obtained from hydrophobic interaction chromatography. The Western blot was detected by anti-α chain staining. It should be noted that DL and NW refer to the "flowthrough" and "wash" fractions, respectively.

Table 14. Typical results of a chromatographic run with 4B3-IgA employing hydrophobic interaction chromatography.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	5.00	275.24	1376.19	-
Flowthrough	17.92	0.14	2.54	0.18
Wash #1	6.28	0.02	0.10	0.01
Wash #2	1.70	0.10	0.18	0.01
Wash #3	7.15	2.52	18.00	1.31
Regeneration	8.06	98.39	793.05	57.63
				Total 59.14

3.6.2 Single step chromatography

Single step chromatography was performed by using the CaptureSelect human IgA affinity resin. This purification strategy allowed the removal of major protein impurities in a single step and gave highly pure IgA eluate fractions. As previously reported, the recovery yields obtained typically ranged between 97% and 96% for 3D6-IgA and 4B3-IgA, respectively (Reinhart et al., 2012).

Figure 42 and Figure 44 show typical chromatograms of 3D6-IgA and 4B3-IgA purification from UF/DF retentate, respectively. The fractions obtained were analyzed by silver staining of SDS-PAGE as well as by Western blotting to evaluate fraction purity and to confirm IgA identity and size (Figure 43 and Figure 45). ELISA was performed to determine antibody titer and recovery yield during the individual chromatographic steps (Table 15 and Table 16).

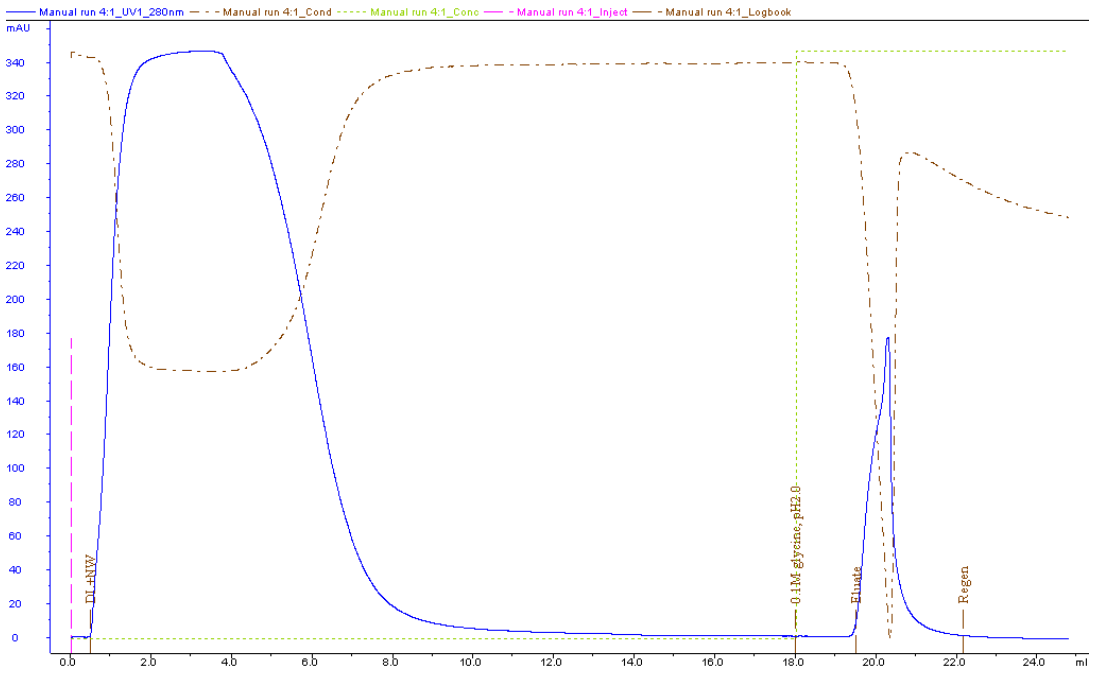

Figure 42. Typical chromatogram of 3D6-IgA purification from cell culture concentrate by CaptureSelect human IgA affinity matrix.

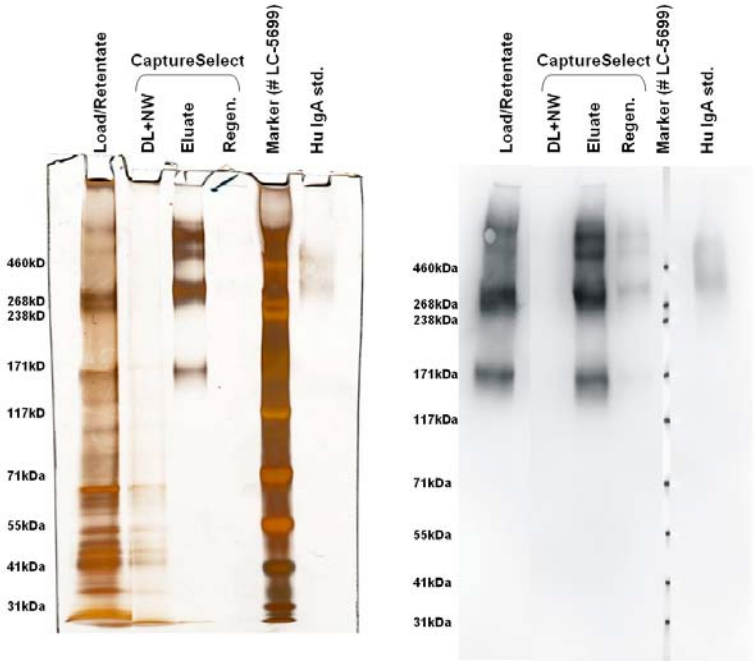

Figure 43. Typical results of an SDS-PAGE silver stain and Western blot with 3D6-IgA fractions obtained from IgA affinity chromatography. The Western blot was detected by anti-α chain staining. It should be noted that DL and NW refer to the "flowthrough" and "wash" fractions, respectively.

Table 15. Typical results of an IgA affinity chromatography experiment with 3D6-IgA.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	5.00	344.55	1722.75	-
Flowthrough/Wash	22.30	2.32	51.74	3.00
Elution	2.86	592.44	1694.38	98.35
Regeneration	3.62	29.45	106.61	6.19
				Total 107.54

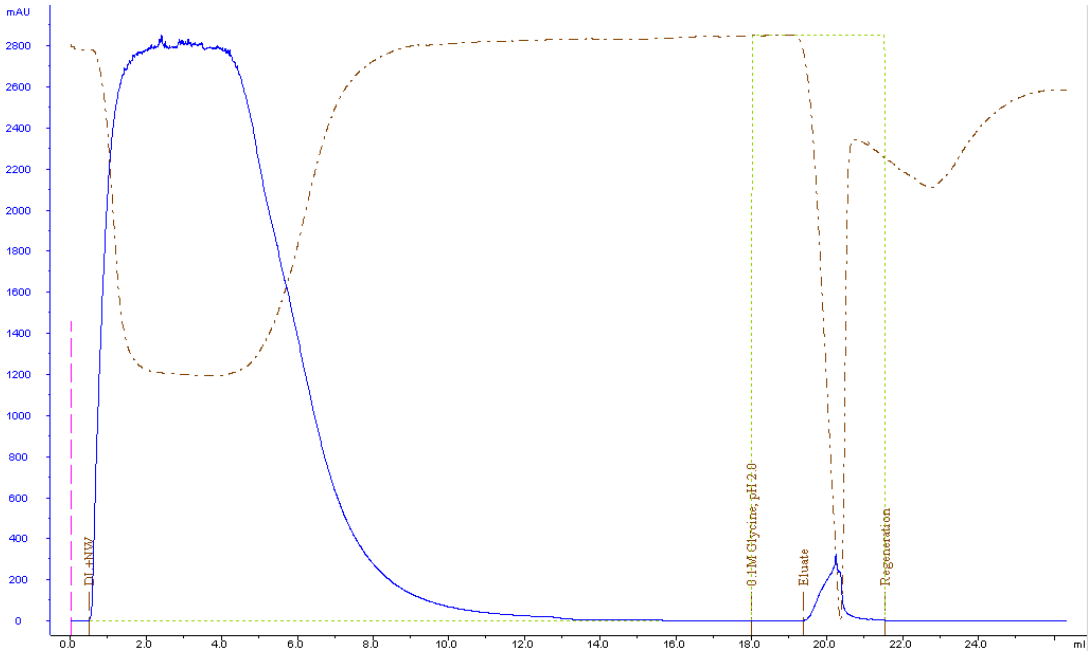

Figure 44. Typical chromatogram of 4B3-IgA purification from cell culture concentrate by CaptureSelect human IgA affinity matrix.

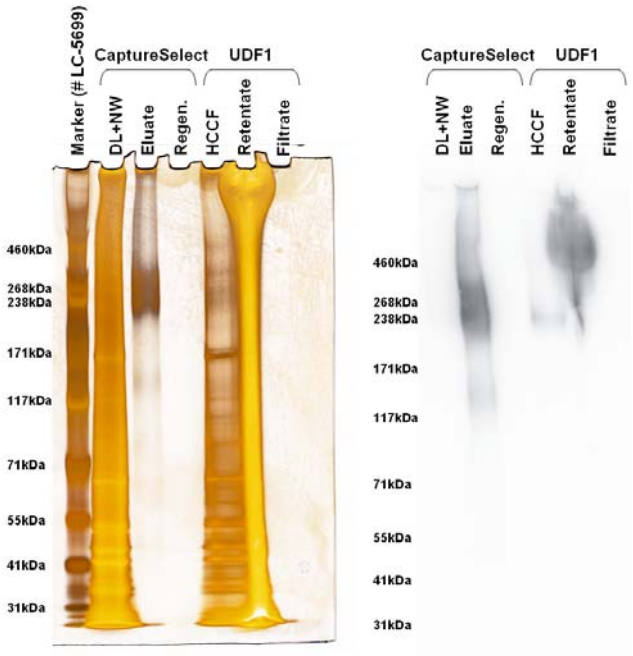

Figure 45. Typical results of an SDS-PAGE silver stain and Western blot with 4B3-IgA fractions obtained from IgA affinity chromatography. It should be noted that DL and NW refer to the "flowthrough" and "wash" fractions, respectively.

Table 16. Typical results of an IgA affinity chromatography experiment with 4B3-IgA.

Fraction	Volume [mL]	Concentration [µg/mL]	Total IgA [µg]	Yield [%]
Load	24.70	103.87	2565.59	-
Flowthrough/Wash	37.62	1.62	60.94	2.38
Elution	5.76	425.95	2453.47	95.63
Regeneration	3.22	4.18	13.46	0.52
				Total 98.53

3.6.3 Comparison of single and multiple step chromatography

In Figure 46 and Figure 47 the eluate fractions after each chromatographic step are shown or a direct comparison of the two chromatographic approaches. While single step chromatography was able to recover IgA in a highly pure fraction after one chromatographic step, several additional steps had to be used in the multiple step chromatography approach. Nonetheless, the purity of the IgA fraction finally obtained was still less pure than that of the single step approach. Furthermore, during the multiple step chromatography approach much more product was lost (52-61% total recovery) than with the single step approach (91-92% total recovery) as shown in Table 17 and Table 18.

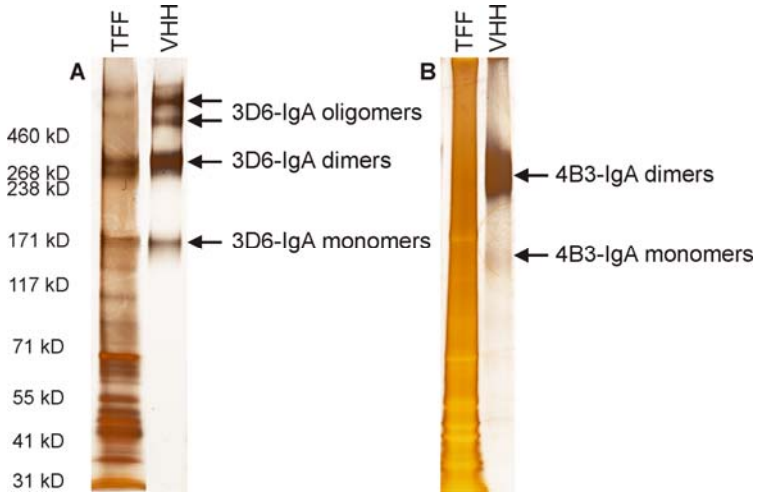

Figure 46. Purity analysis of fractions obtained by single step chromatography. Equal amounts of (A) 3D6-IgA and (B) 4B3-IgA were separated by SDS-PAGE and visualized by silver staining.

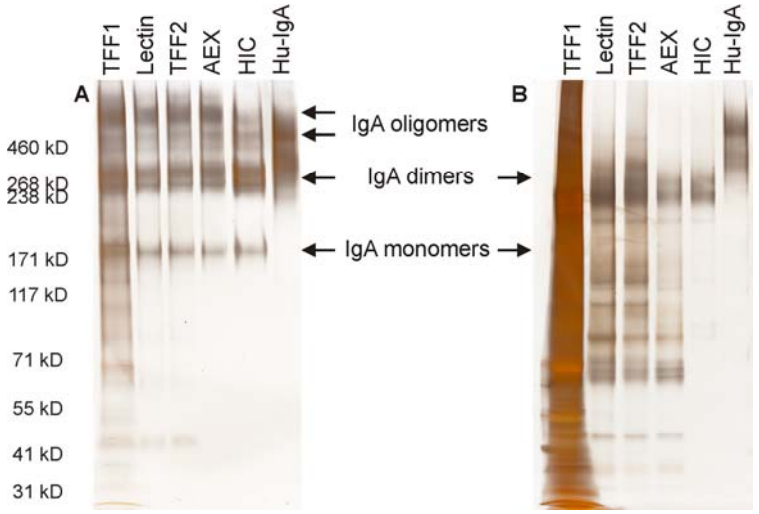

Figure 47. Purity analysis of fractions obtained by multiple step chromatography. Equal amounts of (A) 3D6-IgA and (B) 4B3-IgA were separated by SDS-PAGE and visualized by silver staining.

Table 17. Protein purification of 3D6-IgA and 4B3-IgA by single step chromatography. The yields obtained and purities for each individual step are shown.

Step	Yield (%)		Purity	
	3D6-IgA	4B3-IgA	3D6-IgA	4B3-IgA
UF/DF	95	95	-	--
VHH chromatography	97	96	++++	++++
Total	92	91		

Table 18. Protein purification of 3D6-IgA and 4B3-IgA by multiple step chromatography. The yields obtained and purities for each individual step are shown.

Step	Yield (%)		Purity	
	3D6-IgA	4B3-IgA	3D6-IgA	4B3-IgA
UF/DF	95	95	-	--
Jacalin lectin	97	98	+	+
UDF	95	95	+	+
AEX	87	87	++	++
HIC	77	68	+++	+++
Total	61	52		

4 Discussion

4.1 Influence of transgene on clone performance

RNA is made up of four different nucleotides which can be combined in 64 possible combinations of codons for the 20 different amino acids to be synthesized. Although different synonymous codons encode for the same amino acid it now becomes clear that the chosen codons in a transgene can have varying effects on clone performance.

The usage of synonymous codons can range from frequent to rare and even varies between different genes and genomes. Furthermore, growth conditions and tRNA recharging kinetics may also influence codon usage (Nørholm et al., 2012). “Codon optimization” which is frequently performed for transgenes can have different effects on recombinant protein expression. It has been reported that synonymous codon choice is neutral in most cases. However, changes can influence mRNA stability, mRNA structure, translational initiation, translational elongation and protein folding (Nørholm et al., 2012). Single synonymous codon change in a membrane-bound protease in *E.coli* (ftsH) increased the stability of mRNA structure around the ribosome-binding site, which inhibited translational initiation and reduced protein expression (Makino et al., 1997). Likewise, a synonymous codon change in an *E.coli* outer membrane protein (ompA) lowered the mRNA stability and protein expression 10-fold (Deana et al., 1998). Another research group performed silent mutations in the human mammalian membrane transport protein (P-glycoprotein) and transiently expressed the variants in different mammalian cell lines (HeLa, BSC-1, Vero-76 and 12E1). The change from frequent to rare synonymous codons supposedly altered the timing of translation and co-translational folding which in turn affected protein conformation and substrate specificity (Kimchi-Sarfaty et al., 2007). In contrast, changing of rare-to-frequent synonymous codons of a multidomain protein in *E.coli* (SufI) resulted in an acceleration of the translational rate. As a consequence, alterations in ribosome-mediated translational attenuation led to incorrectly folded proteins despite the same amino acid sequence (Zhang et al., 2009).

In addition to the nucleotide sequence of the transgene itself, regulatory signals in the untranslated regions (UTR) can also control translation, mRNA, localization and expression (Hesketh et al., 1998). In this case, mRNA translation is regulated by controlling translation initiation through formation of a complex between regulatory proteins, mRNA and the small ribosomal subunit. Certain stem-loop structures allow particular cell-specific and mRNA-specific proteins to bind mRNA and govern mRNA translation. Stem loops and other secondary structures at the mRNA’s 5’end appear to inhibit translation. Another important prerequisite for transgene expression is the mRNA’s ability to compete for initiation factors and the phosphorylation of the elongation initiation

factors. Finally, a longer poly(A) tail may result in a more efficient mRNA translation (Hesketh et al., 1998). Therefore, we applied the same plasmids and regulatory sequences for the expression of 3D6-IgA and 4B3-IgA.

It becomes clear that for a clone to perform efficiently, transcription has to go hand in hand with translation. While a limited availability of mRNA will lead to low protein expression rates, a surplus of mRNA with enhanced transcription but a limited ER folding capacity might result in protein aggregation as was recently shown for an IgG antibody in CHO cells (Gomez et al., 2012).

4.2 Influence of primary structure on clone performance

The primary amino acid sequence is fundamental to the biophysical properties of an antibody. The respective sequence ultimately influences the thermodynamic stability and folding efficiency which in turn influences protein solubility and aggregation behaviour (Tiller et al., 2013). Other key factors that are determined by the amino acid sequence are the target epitope, effector functions mediated by the Fc-part, pharmacokinetics as well as immunogenicity.

The importance of even a single amino acid for antibody expression was recently shown when a single amino acid substitution resulted in 80-fold different specific productivities of an IgG antibody (Mason et al., 2012). The fact that dimeric 3D6-IgA and 4B3-IgA show only 72% amino acid sequence homology (84% HC, 42% LC and 100% JC identity) makes their comparison controversial. However, it should be noted that in Mason's work the respective amino acid exchange introduced an endoribonuclease cleavage site into the mutant's nucleotide sequence which reduced the amount of available translatable mRNA in the mutant. Therefore, it is possible that not only the amino acid substitution itself was responsible for the different IgG expression levels. However, in a direct comparison a destabilizing effect of the substituted amino acid on the mutant's Fab domain was shown (Mason et al., 2012). If such mutations constrain post-translational events such as folding and a proper orientation of the VH/VL dimer, a correct HC and LC pairing can be impeded and antibody production challenged.

It is well-known that low protein expression levels can result from sub-optimal folding properties (Schaefer and Plückthun, 2012). The efficiency of polypeptide folding and antibody assembly therefore predominantly depends on the amino acid sequence present. Recently, there has been growing interest in discovering naturally occurring and optimally fitting antibody HC and LC pairings, which are thought to lead to higher productivities. If folding does not allow a proper VH/VL dimer orientation the association of LC with HC, a prerequisite for whole antibody secretion, is impaired. Tiller et al. (2013)

have constructed a fully synthetic human Fab antibody phage display library which was built on 36 fixed germline VH/VL framework pairs. It was shown that there are some natural preferences in pairing of particular VH and VL gene families. Furthermore, the combination of different VH/VL pairs resulted in alternating protein expression, thermal stability and aggregation (Tiller et al., 2013). Unfortunately, the VH/VL combination for 3D6-IgA (IGHV3-9/IGKV1-5) and 4B3-IgA (IGHV1-69/IGLV6-57) was not analyzed, which could have helped to understand potential differences in their biophysical pairing properties.

The primary amino acid sequence chiefly determines a protein's fold and therefore its compactness and stability. Stability is defined as a protein's resistance to denaturation in response to environmental stress such as temperature, proteolytic degradation or denaturing agents. There are numerous cases in which a protein's thermal stability was correlated with chemical stability and protease resistance (Willuda et al., 1999). Furthermore, stable proteins often have a reduced tendency for aggregation and are frequently expressed at higher concentrations (Traxlmayr and Obinger, 2012). For antibodies, especially, the variable domains vary widely in their intrinsic thermodynamic stability. It has been found that in scFv the VL and VH domain interaction is mutually stabilizing and depends on the stability of the individual domains and the strength of the interface. As a result, the interaction between two weak V domains can give rise to a significantly more stable complex than either of the domains themselves (Honegger, 2008). Therefore, novel techniques aim to engineer suboptimal V domains for improved stability and folding efficiency.

If HC fails to associate with LC the formation of intracellular inclusions of aggregated immunoglobulins, so-called Russell bodies (RBs), might occur. RBs, which are described as cisternal dilations of the endoplasmic reticulum, are generally considered to appear due to protein folding and dislocation insufficiencies of the ER machinery (Kopito and Sitia, 2000). Since LC is able to leave the ER in an unbound state, but HC can only be secreted when it is associated with LC (Lee et al., 1999a), LC has been proposed to be of key importance for RB generation (Corcos et al., 2010). In a more recent publication RB induction has been suggested to depend on the intrinsic properties embedded in the VL domain and its compatibility with the partnering VH domain sequence. Stoops et al. (2012) has shown that LCs with different V domains possess competence differences to facilitate HC folding and IgG assembly. The co-expression of LCs derived from poorly secreting clones with HCs from high secreting clones led to titer reductions of 25-90%. In contrast, LCs from high secreting clones co-expressed with poorly secreting HCs increased IgG titers up to two orders of magnitude and significantly reduced RB formation (Stoops et al., 2012). The authors argued that the high competence of LC alone is not

sufficient to attain highly efficient IgG assembly, but is limited to the compatibility between VH and VL domains. In the context of our results the question arises: what was the reason for RB appearance in 4B3-IgA, but not in 3D6-IgA? In general, the human VH3 domain (3D6) has the best biophysical properties among human subtypes and is by far the most stable VH domain, followed by VH1 (4B3), (Honegger, 2008). Furthermore, the VH/VL pairing in the natural human antibody repertoire of IGHV3-9/IGKV1-5 (3D6) and IGHV1-69/IGLV6-57 (4B3) is rather frequent and therefore nothing “exotic” (Tiller et al., 2013). Since HC aggregated despite the presence of LC in 4B3-IgA (but not 3D6-IgA) we believe that the folding competence of 4B3 LC was not sufficient for an efficient HC folding and IgA assembly. HC, which needs LC to fold (Lee et al., 1999a) aggregated and was deposited as RBs. Bottlenecks in the ER folding machinery were further highlighted by the upregulation of KDEL-containing chaperones in 4B3-IgA which were believed to assist the low folding competence of 4B3 LC.

It is known that lowering the temperature exerts a nonspecific, folding-promoting effect presumably by stabilizing native structures or by reducing aggregation (Ellgaard and Helenius, 2003). To test this, we cultivated the recombinant cell lines at lower temperatures (data not shown). Propagation of the 3D6-IgA cell line at 35°C doubled the titer from 90 µg/mL (37°C) to 210 µg/mL and 3-fold to 280 µg/mL at 33°C. This temperature effect was even more pronounced for 4B3-IgA where cultivation at 35°C increased the titer 5-fold from 0.7 µg/mL (37°C) to 3.5 µg/mL and even 15-fold to 10.0 µg/mL at 33°C. These results further suggested that 4B3-IgA could not fold HC and assemble with LC at standard temperatures easily. However, folding and antibody expression was facilitated at hypothermic conditions. It is probable that the lower temperatures led to a reduced exposure of hydrophobic amino acids, which might have decreased aggregate formation as well as binding of ER-retaining chaperones (Ellgaard and Helenius, 2003).

4.3 Novel strategies for IgA purification

The different amounts of host cell protein impurities, IgA content and isoforms in the culture supernatants of 3D6-IgA and 4B3-IgA qualified them as interesting tools for the evaluation of the purification process. State-of-the-art antibody purification is typically initiated by centrifugation and filtration of the fermentation broth after its harvest from the bioreactor. Then, the product is further concentrated and clarified via a high affinity capture step. Product polishing is achieved through one or two subsequent chromatographic steps (Kelley, 2009).

Unlike the enabling bioaffinity capture method that permitted the commercial success of more than two dozen IgGs, there is no generically applicable IgA purification procedure. It

is well known that Protein A binds to human VH3 encoded IgAs via the variable region (Grov, 1975; Grov, 1976), but in the 35 years since that discovery, there has been no report of Protein A being seriously considered as a general IgA purification tool. In literature, the α-D-galactose binding lectin termed immobilized jacalin is frequently employed as a primary IgA1 capture step. In our experiments, immobilized jacalin yielded high product recoveries for both antibodies and removed substantial amounts of impurities, especially from 4B3-IgA UF/DF retentates. Nevertheless, the eluate fractions still contained high amounts of host cell protein, which highlights one of several weaknesses of this chromatography technique i.e. co-elution of proteins with a glycosylation pattern similar to IgA1 (Roque-Barreira and Campos-Neto, 1985). Furthermore, its inability to bind IgA2 reduces the applicability of immobilized jacalin as a generic IgA capture step. Another disadvantage was found when culture supernatants were concentrated >60-fold which impaired IgA capture and decreased yields considerably (data not shown). From the point of view of the process being economic this would result in an extended purification duration and product-hold time. However, in our experiments immobilized jacalin reduced the amount of impurities impressively, even from highly impure protein samples such as 4B3-IgA, and served as a good starting point for subsequent purification steps. With later multiple chromatographic techniques an acceptable purity was obtained, however, at the expense of product.

Other possible techniques for IgA purification, but which were not performed in this work, might be the application of Protein L which specifically recognizes the framework region 1 of certain kappa light chains. However, this resin material suffers from several limitations since it does not bind human kappa light chains of subtype II as well as antibodies with lambda light chains (Boes et al., 2011). Furthermore, the purification success of this technique for non-recombinant samples such as biologic serum or mucosal secretions remains highly questionable.

More recently, camelid VHH ligands have been promoted as affinity ligands for a variety of challenging purification applications. VHH ligands are 12-15 kDa single-domain ligands that lack any light chain as well as the CH1 domain of conventional antibodies. Their unique stability and specificity has been shown successfully for the chromatographic purification of different molecules (Detmers et al., 2010). The anti-human alpha-chain VHH ligand which we used in VHH affinity chromatography recognizes a unique domain on human IgA and all its subclasses. IgA purification without cross-reactivity with IgG or IgM renders this technique a powerful tool. In our experiments VHH chromatography was outstanding in terms of purities and yields. In a single step both IgAs were recovered with yields >95% and exceptionally high purities. Nevertheless, the manufacturer's recommendation for VHH chromatography was not applicable for our IgAs since an initial

elution with 0.1 M glycine at pH 3 yielded only 40 % recovery (data not shown). Lowering the pH to 2 improved results considerably. The new elution pH was not considered to impair IgA stability as IgAs are naturally present in the gut at comparable pH values (McClelland et al., 1972). However, to obtain certainty we tested product stability SDS-PAGE in which IgA bands appeared at the same positions before and after elution without any observable degradation. Also, the intact immunoreactivity of both IgAs to a recombinant HIV-1 trimeric glycoprotein (CN54 gp140) was confirmed by ELISA (data not shown).

4.4 Assembly of sIgA

Secretory IgA is the principal immunoglobulin in secretions of mucosal membranes found in the gastrointestinal, respiratory and genitourinary tracts. *In vivo*, sIgA is produced by two distinct cell types. First, antibody-secreting plasma cells within the mucosae and exocrine cells assemble IgA HC, LC and JC to produce polymeric IgA. In a second step, pIgA binds to the pIgR expressed on the basolateral side of epithelial cells lining the mucosae. Upon transcytosis the extracellular portion of pIgR (SC) remains attached to pIgA to be secreted as sIgA.

Dimeric IgA and SC are mutually protective polypeptides, which increase the protease resistance of the formed sIgA complex. This was demonstrated by Crottet and Corthésy (1998) who incubated dIgA with mouse intestinal washes and showed that 10-fold more concentrated washes were necessary to reach the same proteolytic effect on sIgA. Free SC was completely digested after an incubation period of 30 minutes, highlighting its mutually protective role with dIgA. The α-chain digestion patterns of dIgA and sIgA were remarkably similar. The authors suggested that the same sensitive sites remain exposed in dIgA and sIgA, whereas small structural changes in bound SC give temporary access to proteases. Similar results for the resistance to proteolytic digestion of sIgA compared to polymeric IgA were reported by Berdoz et al. (1999).

Our initial aim was to produce sIgA by co-expressing HC, LC, JC and hSC polypeptides in a single recombinant CHO cell. Berdoz et al. (1999) have shown that such an approach is feasible. Our experiments yielded poor antibody titers (especially for 4B3-sIgA) and a random assembly of polypeptides (for 3D6-sIgA). In an alternative approach we aimed to produce sIgA by expressing hSC with a separate cell line and use it for an *in vitro* association of hSC with polymeric IgA. The generated transfectants produced hSC with appropriate size (80 kDa) and sufficient quantities with titers up to 40 µg/mL. Next, we tested the *in vitro* association of hSC with polymeric IgA at different molar ratios and temperatures. Most reports suggest that the optimal molar ratio for sIgA assembly is 1:1 (pIgA:hSC) at ambient temperature (Rindisbacher et al., 1995; Lüllau et al., 1996; Crottet

and Corthésy, 1998). Crottet and Corthésy (1998) showed that at ambient temperature (15°C, 22°C and 37°C) a plateau for sIgA assembly was reached typically after 1 hour of incubation which faintly increased within the following 15 hours. Although sIgA yields never reached 100% (even with higher molar ratios of pIgA:SC), roughly 80% of dIgA was covalently associated with SC, whereas 20% was bound in a non-covalent way. Incubation at 4°C reduced the formation of covalent complexes from 80% to 30-35%. Finally, the addition of redox agents such as DTT and glutathione or a disulfide-interchange enzyme (PDI) did not visibly improve the covalent association of SC and pIgA. Our results showed that higher temperatures and molar ratios improved sIgA yields. In terms of temperature, it is probable that slightly elevated temperatures modified the protein conformations in a way that pIgA and hSC interaction was facilitated. In terms of molar ratio, it is plausible that a higher molarity of hSC increased the chance of encountering a pIgA molecule and therefore reached a saturation plateau earlier than lower hSC molarities. If this is the case, the plateau for sIgA assembly had not yet been reached and a prolonged incubation time, as suggested by Lüllau et al. (1996), might balance the yields in future experiments.

5 Conclusions and perspective

Besides a few other mammalian cell lines, Chinese hamster ovary (CHO) cells remain the dominant choice as the expression system for more than 70% of protein pharmaceuticals on the market (Jayapal et al., 2007). Recombinant IgAs are not yet therapeutically available but are progressively gaining interest due to their unique role in mediating mucosal immunity. However, the production of adequate amounts has two major bottlenecks i.e. the generated transfectants often yield low IgA titers and no generically applicable purification procedure has yet been found.

To meet the requirements of complex proteins such as IgA the respective production system needs to be properly investigated. Protein production typically involves the transcription of the relevant exogene(s), mRNA transport from the nucleus to the membrane of the endoplasmic reticulum (ER) for translation initiation and protein translocation into the ER, followed by N-glycosylation, folding and assembly of light chains (LC) with heavy chains (HC) and even a J chain (JC) in case of polymeric IgA and IgM. Correctly processed molecules will be forwarded to the Golgi apparatus for further modifications and finally be secreted. Any of these steps is crucial and may be decisive in whether a clone will become a high producing cell line or not. High-level protein expression remains controversial and is often reported to coincide with high gene copy numbers (GCN) and mRNA levels (Schröder et al., 1999; Jiang et al., 2006). However, other reports claim that GCN does not necessarily correlate with antibody expression (Lattenmayer et al., 2007; Reisinger et al., 2008) and clones with the highest mRNA levels are not always those with the highest productivity (Chusainow et al., 2009). Mohan et al. (2008) has shown that an increased GCN or mRNA level was irrelevant for the secreted protein amount and therefore suggested that the bottleneck in enhancing productivity is a translational or post-translational process. Our results revealed high protein expression for 3D6-IgA, but low titers for 4B3-IgA despite the fact that no major differences in GCN and mRNA levels could be observed. The amino acid sequence homology of the two antibodies comprised 72% which may result in differences in intracellular protein processing and makes a comparison controversial. Since GCN and mRNA most probably did not cause any restrictions in IgA productivity a bottleneck in post-translational processing seemed likely.

Among the post-translational steps, the correct assembly of HC and LC has been proposed to be the major limiting factor for antibody production (Bibila and Flickinger, 1992). Furthermore, the correct pairing of VH/VL domains has been proposed to be essential for high antibody expression (Honegger, 2008; Tiller et al., 2013). Incompletely assembled and unfolded HC is bound by BiP (Heavy Chain Binding Protein or GRP78)

(Bole et al., 1986), recognized as an immature glycoprotein by the lectin chaperones calnexin and calreticulin and retained in the ER (Helenius and Aebi, 2004). Polypeptides that cannot be refolded/assembled are removed from calnexin/calreticulin by ER mannosidase I and ER degradation-enhancing mannosidase-like proteins and are eliminated by the ubiquitin-proteasome system by means of ER-associated degradation (Davies et al., 2011). Our results indicated that polypeptide assembly and 3D6-IgA production was efficient since high titers with correctly folded antibodies were determined. On the contrary, 4B3-IgA production was found to be challenged by several means: first, intracellular aggregates of mostly HC polypeptides were found during fluorescence microscopy. These so-called RB deposits were formed despite the presence of LC, which was shown to have a decisive role as its proper assembly with HC will trigger the release from BiP and thereby facilitates HC to fold correctly (Lee et al., 1999b). Second, Western blotting of intracellular product showed that the polypeptides which constitute 4B3-IgA molecules were often not properly assembled and resulted in aggregate formation and supposedly was the cause for the low productivity observed in the respective cell line. Only a small fraction of 4B3-IgA was correctly folded/assembled and secreted into the culture supernatant. Third, using a KDEL-specific antibody during flow cytometry allowed us to correlate the resulting ER stress in the protein machinery to bottlenecks in 4B3-IgA processing. For future clone development it may be beneficial to include screening of ER stress signals in addition to HC/LC content. It remains to be shown if the low productivity of 4B3-IgA was due to a suboptimal pairing of their V domains (Honegger, 2008; Tiller et al., 2013). From what is known 3D6-IgG expresses high titers, whereas 4B3-IgG is a poor secreting cell line, similar to their IgA isotype variants (personal communication with Prof. Kunert). Novel techniques such as engineering V domains for improved stability and folding efficiency may improve antibody titers and gain additional understanding.

We showed that the assembly of polymeric IgA with hSC to form sIgA was possible. Considering the protective role of hSC against proteolysis in mucosal tissues (Crottet and Corthésy, 1998), the equipment of our polymeric IgAs with hSC might be a tremendous improvement for resistance and half life especially for the planned HIV challenge experiments *in vivo* with macaques. However, the assembly of hSC with 4B3-IgA (which was mostly dimeric) was more efficient than with 3D6-IgA (which was di-, tri- and tetrameric). It appeared, at least in our experiments, that a higher degree of IgA polymerization opposes a steric hindrance for hSC accession and sIgA formation.

For IgA purification, to date there is still no generically applicable downstream scheme such as the enabling bioaffinity capture method that permitted the commercial success of more than two dozen IgGs. In order to address this issue we developed two different

approaches. The cell culture supernatants of the high (3D6-IgA) and low (4B3-IgA) were considered to be a well-representative study tool due to their difference in IgA content, the relative difference in the amount of protein impurities as well as the different forms of IgA isoforms. In the first approach, a multistep purification process was developed which consisted of an affinity step based on immobilized jacalin followed by anion exchange and hydrophobic interaction chromatography. Despite relatively high total product losses of 52-61% satisfyingly pure IgA fractions could be obtained. In the second approach we employed a VHH ligand and isolated both recombinant IgAs at even higher purities and total yields of 91-92% in a single chromatographic step. Furthermore, unlike the first approach, VHH chromatography was irrespective of the antibody light chain and specificity. In addition to its high purification potential and recovery yields the method described promises to be powerful as a generic capture step for the isolation of any IgA.

6 References

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7 List of tables

8 List of figures

9 Selected publications

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10 Appendix

10.1 Nucleotide sequences of used plasmids

10.1.1 pMG433_3D6IgA_LC

GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCAC
GACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCAC
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10.1.2 pMG433_4B3IgA_LC

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10.1.8 pIRES(AB)dhfr_hSC_3D6-IgA-HC

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10.1.9 pIRES(AB)dhfr_hSC_4B3-IgA-HC

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10.1.11 pIRES(AB)neo_4B3LC_JC

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CTATATGTGATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCC
TGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTT
GAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGA
CCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACG
TGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTG
TGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAG
GTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTC
GAGGTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACAC
GATGATAAGCTTGCCACAACCCGGGATCCT

10.2 Amino acid sequences of expressed polypeptides

10.2.1 3D6 LC

MALQTQVFISLLLWISGAYGDIQMTQSPSTLSASVGDRVTITCRASQSISRWLAWYQQKPGKVPK
LLIYKASSLESGVPSRFSGSGSGTEFTLTISSLQPDDFATYYCQQYNSYSFGPGTKVDIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS
TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

10.2.2 3D6 IgA HC

MDWTWRILFLVAAATGVHSEVQLVESGGGLVQPGRSLRLSCAASGFTFNDYAMHWVRQAPGKG
LEWVSGISWDSSSIGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDMALYYCVKGRDYYDSGGY
FTVAFDIWGQGTMVTVSSASPTSPKVFPLSLCSTQPDGNVVIACLVQGFFPQEPLSVTWSESGQ
GVTARNFPPSQDASGDLYTTSSQLTLPATQCLAGKSVTCHVKHYTNPSQDVTVPCPVPSTPPTP
SPSTPPTPSPSCCHPRLSLHRPALEDLLLGSEANLTCTLTGLRDASGVTFTWTPSSGKSAVQGPP
ERDLCGCYSVSSVLPGCAEPWNHGKTFTCTAAYPESKTPLTATLSKSGNTFRPEVHLLPPPSEEL
ALNELVTLTCLARGFSPKDVLVRWLQGSQELPREKYLTWASRQEPSQGTTTFAVTSILRVAAEDW
KKGDTFSCMVGHEALPLAFTQKTIDRLAGKPTHVNVSVVMAEVDGTCY

10.2.3 4B3 LC

MALQTQVFISLLLWISGAYGNSMLTQPHSVSESPGKTVTISCTRTSGSIDTNYVQWYQQRPGSAP
TTVIFEDNQRPSGVPDRFSGSIDSSSNSASLTISGLQTEDEADYYCQSYGTNNHGVFGGGTKLTV
LGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNN
KYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS

10.2.4 4B3 IgA HC

MDWTWRILFLVAAATGVHSQVQLQESGAEVKTPGSSVKVSCKAFGGTHHNYAINWVRQAPGQG
LEWMGGIVPILGIASYTQKFQGTVALTADKSTNTAYMELTSLRSEDTAVYFCAAFCLSPRCSRGYY
AMDVWGQGTTVIVSSASPTSPKVFPLSLCSTQPDGNVVIACLVQGFFPQEPLSVTWSESGQGVT
ARNFPPSQDASGDLYTTSSQLTLPATQCLAGKSVTCHVKHYTNPSQDVTVPCPVPSTPPTPSPST
PPTPSPSCCHPRLSLHRPALEDLLLGSEANLTCTLTGLRDASGVTFTWTPSSGKSAVQGPPERDL
CGCYSVSSVLPGCAEPWNHGKTFTCTAAYPESKTPLTATLSKSGNTFRPEVHLLPPPSEELALNE
LVTLTCLARGFSPKDVLVRWLQGSQELPREKYLTWASRQEPSQGTTTFAVTSILRVAAEDWKKG
DTFSCMVGHEALPLAFTQKTIDRLAGKPTHVNVSVVMAEVDGTCY

10.2.5 J chain

MKNHLLFWGVLAVFIKAVHVKAQEDERIVLVDNKCKCARITSRIIRSSEDPNEDIVERNIRIIVPLNN
RENISDPTSPLRTRFVYHLSDLCKKCDPTEVELDNQIVTATQSNICDEDSATETCYTYDRNKCYTA
VVPLVYGGETKMVETALTPDACYPD

10.2.6 Human secretory component

MLLFVLTCLLAVFPAISTKSPIFGPEEVNSVEGNSVSITCYYPPTSVNRHTRKYWCRQGARGGCIT
LISSEGYVSSKYAGRANLTNFPENGTFVVNIAQLSQDDSGRYKCGLGINSRGLSFDVSLEVSQGP
GLLNDTKVYTVDLGRTVTINCPFKTENAQKRKSLYKQIGLYPVLVIDSSGYVNPNYTGRIRLDIQGT
GQLLFSVVINQLRLSDAGQYLCQAGDDSNSNKKNADLQVLKPEPELVYEDLRGSVTFHCALGPEV
ANVAKFLCRQSSGENCDVVVNTLGKRAPAFEGRILLNPQDKDGSFSVVITGLRKEDAGRYLCGAH
SDGQLQEGSPIQAWQLFVNEESTIPRSPTVVKGVAGGSVAVLCPYNRKESKSIKYWCLWEGAQN
GRCPLLVDSEGWVKAQYEGRLSLLEEPGNGTFTVILNQLTSRDAGFYWCLTNGDTLWRTTVEIKII
EGEPNLKVPGNVTAVLGETLKVPCHFPCKFSSYEKYWCKWNNTGCQALPSQDEGPSKAFVNCD
ENSRLVSLTLNLVTRADEGWYWCGVKQGHFYGETAAVYVAVEERKAAGSRDVSLAKADAAPDE
KVLDSGFREIENKAIQDPRLFAE

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Research paper

Recombinant IgA production: Single step affinity purification using camelid ligands and product characterization

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ABSTRACT

Immunoglobulins of isotype A (IgA) mediate a key role in mucosal immunity and are promising new immunotherapeutic candidates, but difficulties in obtaining enough material often hamper their *in vivo* exploration. We established recombinant Chinese hamster ovary (CHO) cells which stably expressed two IgA1 antibodies under serum-free conditions. The two cell lines achieved significantly different specific productivities of 16 pg per cell and day and 100 times less, a common phenomenon in recombinant antibody expression which challenges the production and purification process. Polymeric IgA in crude culture supernatants was assembled with J chain and showed expected specificity. We employed an immobilized camelid anti-human alpha-chain VHH ligand and isolated both recombinant IgAs at high purity and yield in a single chromatographic step. The described method was irrespective of the light chain and specificity and may be used as a generic capture step for the isolation of any IgA. Results were compared with a multistep purification process consisting of an affinity step based on immobilized jacalin followed by anion exchange and hydrophobic interaction chromatography.

1. Introduction

The estimated market value of recombinant therapeutic antibodies rapidly approached $43 billion in 2010 (Elvin et al., 2011), but not one of those is an IgA. This is remarkable because IgAs have a unique role in mediating mucosal immunity and could foster the treatment or prevention of a variety of diseases. This highlights an important bias in the way the field of immunotherapy has developed: clinical candidates are chosen more on the basis of enabling purification technology than on the potential clinical merits of the antibody.

To date, IgA has lacked the enabling bioaffinity capture method that permitted the commercial success of more than two dozen IgGs. Protein A has been documented to bind human VH3 encoded IgAs via the light chain variable region (Grov, 1975, 1976), but in the 35 years since that discovery, there has been no report of Protein A being seriously considered as a general IgA purification tool. IgA purification is sometimes performed using immobilized jacalin, an α-D-galactose binding lectin, which only binds IgA1 but not IgA2. The usefulness of immobilized jacalin for IgA1 purification is limited, because lectins are additionally accessible to similarly glycosylated proteins which co-elute as impurities with the product (Roque-Barreira and Campos-Neto, 1985). Alternative strategies rely on Protein L which specifically recognizes the framework region 1 of certain kappa light chains, but does not bind human kappa light chains of subtype II and antibodies with lambda light chains (Boes et al., 2011). More recently, camelid VHH ligands have been promoted as affinity ligands for a variety of challenging purification applications. VHH ligands are 12–15 kDa single-domain ligands that lack any light chain and the CH1 domain of conventional antibodies. Due to

Abbreviations: CHO, Chinese hamster ovary;DHFR, dihydrofolate reductase;IgA, immunoglobulin A;MTX, methotrexate;TFF, tangential flow filtration

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their unique stability and specificity, VHH ligands have been successfully used for the chromatographic purification of different molecules (Detmers et al., 2010).

Here we describe the development of recombinant CHO production cell lines which secrete two different human IgA molecules together with the human J chain. The antibodies are directed against the gp41 envelope protein of HIV-1 and were originally of human IgG1 subtype (Grunow et al., 1988; Buchacher et al., 1994). A camelid anti-human alpha-chain specific ligand was employed in VHH chromatography to isolate IgAs with kappa (3D6) or lambda (4B3) light chains from cell culture supernatants at high purity and recovery yield in a single step. Results were compared to an alternative IgA purification strategy based on immobilized jacalin, anion exchange (AEX) and hydrophobic interaction chromatography (HIC).

2. Materials and methods

2.1. Generation of expression plasmids

3D6 and 4B3 cDNAs of heavy and light chain variable regions were isolated from hybridoma cell lines (Felgenhauer et al., 1990; Kunert et al., 1998). The cDNA sequence for human IgA1 heavy chain (J00220), kappa light chain (J00241) and lambda light chain (J00253) constant regions as well as J chain (NP653247) can be retrieved online via GenBank. All cDNAs were codon optimized for expression in CHO cells and synthesized by GeneArt AG (Germany). Coding regions were under control of SV40 promoters and expression vectors were constructed to combine heavy chain with the dhfr selection/amplification marker (Wolbank et al., 2003) and J chain with neomycin (Fig. 1).

2.2. Host cell line, transfection, selection and gene amplification

Dihydrofolate reductase (dhfr) deficient CHO host cells, ATCC CRL-9096 (Urlaub and Chasin, 1980), were adapted to grow under protein-free conditions in a DMEM/Ham's F12 1:1 (PAA, Austria) formulation with 4 mM L-glutamine, 0.1 mM hypoxanthine, 0.016 mM thymidine, 0.25 g/l soy peptone, 0.1% Pluronic F-68 and a protein-free supplement (Polymun Scientific GmbH, Austria). DNA/PEI polyplexes were formed of 250 μg PEI (linear, MW: 25,000; Polysciences Inc., PA) with 25 μg plasmid DNA containing J chain, light chain and heavy chain in a ratio of 1:1:1 and used for transfection of 5×10^6 cells (Reisinger et al., 2009). After 24 h selection was started with ProCHO5 (Lonza, Switzerland) supplemented with 4 mM L-glutamine, 15 mg/l phenol red and 0.5 mg/ml G418. Transfectants were seeded at 1×10^5 cells/ml in five 96-well plates. In two transfection experiments a total of 960 wells were generated for each IgA variant. Gene amplification was initiated with 0.05 μM methotrexate (MTX) in selection medium and best clones were identified by ELISA screening. The best 5% producing wells were adapted to 0.1 μM MTX and one clone was selected for limited dilution subcloning to obtain a monoclonal cell line. Clones were fed with 0.4 μM MTX amplification medium and selected as described before. Finally, the best clone was subcloned a third time using the same approach.

2.3. Stability of cell lines

Antibody-containing supernatants were generated by cultivation of recombinant cell lines at 37 °C in spinner vessels at 50 rpm in 50 ml amplification medium. Cells were split every 3–4 days and re-seeded at 2.5×10^5 cells/ml. The total cell number was quantified using a Coulter Counter Z2 (Beckman Coulter, CA) and cell viability was determined by trypan blue dye exclusion. Recombinant cell lines were cultured for at least 10 passages to monitor growth rate and stability of product expression. Specific productivities (q_P) were calculated as picogram mAb per cell and day (pcd) and averaged from all passages in spinner flasks.

2.4. Characterization of IgAs

IgA was quantified by sandwich ELISA using a goat anti-human IgA α-chain specific antiserum (Sigma, MO) for coating and goat anti-human κ-chain (Sigma, MO) together with mouse anti-human λ-chain specific antiserum (Southern Biotech, AL), both peroxidase-conjugated, for detection. IgA standard from human colostrum (Sigma, MO) was adjusted to 400 ng/ml and diluted in 1:2 series together with samples.

Purity and molecule size distribution of purified IgA fractions was determined using NuPAGE Novex 3–8% Tris-

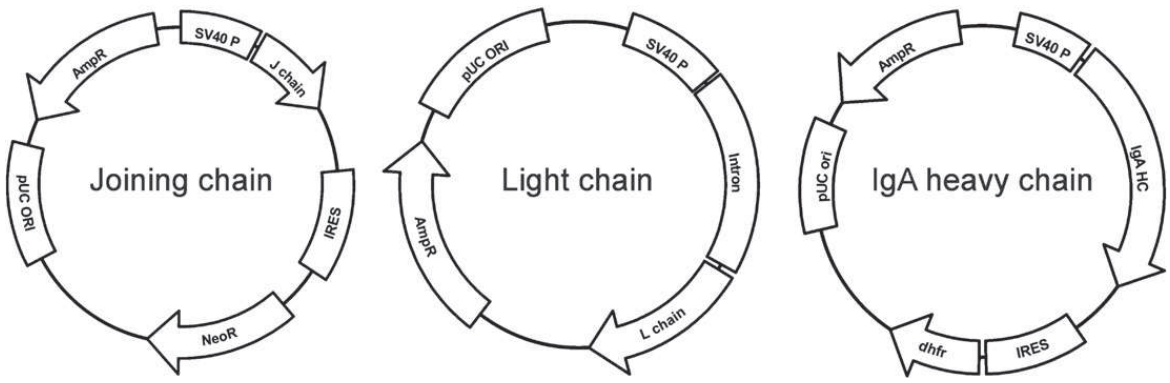

Fig. 1. Plasmids used for the co-transfection of dhfr-deficient CHO host cell line.

Acetate gels purchased from Life Technologies, CA (Vorauer-Uhl et al., 2010) which were silver stained (Heukeshoven and Dernick, 1985). IgA isoforms of 3D6-IgA culture supernatants or 4B3-IgA concentrates were analyzed on Western blots with the same antibodies used in ELISA. J chain was verified via a rabbit anti-human J chain antiserum (Antibodies-online, Germany) and a peroxidase-conjugated anti-rabbit IgG polyclonal serum (Sigma, MO).

2.5. IgA purification from cell culture supernatants

Buffers were freshly prepared and salts were obtained from Sigma, MO. Cell culture supernatants were centrifuged, 0.22 μm filtered and concentrated to 0.5 mg/ml mAb applying a Labscale Tangential Flow Filtration (TFF) System (Millipore, MA) equipped with a KvickStart cassette (30 kDa; GE Healthcare, UK) combined with buffer exchange into 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (PBS). Recombinant IgA was purified by single step and multiple step chromatography, as depicted in Fig. 2. All chromatography experiments were conducted on an ÄKTA Purifier (GE Healthcare, UK) at a linear flow rate of 150 cm/hr (0.5 mL/min) if not indicated differently.

Immobilized anti-human alpha-chain VHH (CaptureSelect IgA) was obtained from BAC, Netherlands. 0.6 mL was packed in a Tricorn 5/100 (GE Healthcare, UK) column and equilibrated with PBS. Sample was loaded then the column washed with PBS prior to elution with 0.1 M glycine, pH 2.0.

Immobilized jacalin (3.2 ml; Thermo Fisher Scientific, MA) was packed into HR16/5 columns (GE Healthcare, UK). The column was equilibrated with PBS before and after sample application. Product was eluted with 1.5 M α-D-galactose in PBS.

For AEX, HR 16/5 columns were filled with 3.3 ml DEAE Sepharose Fast Flow (GE Healthcare, UK). Eluate from lectin chromatography was buffer exchanged into 20 mM Tris, 10 mM NaCl, pH 8.5 by TFF and loaded onto the column. After washing with 10 mM and then 100 mM NaCl in 20 mM Tris, pH 8.5, product was eluted with 20 mM Tris, 200 mM NaCl, pH 8.5.

Phenyl Sepharose FF low sub was obtained from GE Healthcare, UK. 1.9 ml was packed into a Tricorn 5/100 column (GE Healthcare, UK) and equilibrated with 1 M or 0.75 M $(NH_4)_2SO_4$ in 20 mM Tris, pH 7.5 at a linear flow rate of 75 cm/hr for 3D6-IgA or 4B3-IgA, respectively. 3 M $(NH_4)_2SO_4$, pH 8.5 was added to AEX eluates to a final concentration of 1 M or 0.75 M $(NH_4)_2SO_4$ for by-product precipitation. The column was washed with 0.5 M or 0.2 M $(NH_4)_2SO_4$, pH 7.5 for 3D6-IgA or 4B3-IgA after sample loading and eluted with 0.3 M or without $(NH_4)_2SO_4$, pH 7.5, respectively.

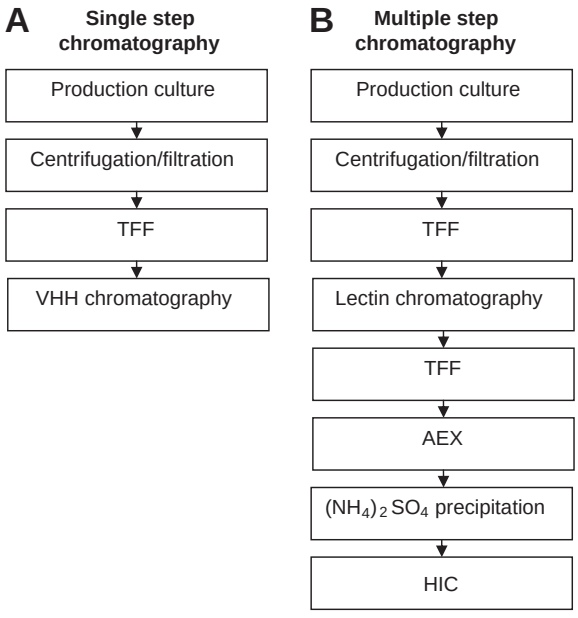

Fig. 2. Process flow for IgA purification from crude cell culture supernatants by (A) single step chromatography and (B) multiple step chromatography.

3. Results

3.1. Recombinant CHO cell lines and IgA production

Specific productivities of 3D6-IgA transfectants ranged between 2.4 ± 0.9 and 6.2 ± 2.3 pcd (Fig. 3A). Increase of MTX to 0.4 μM along with a second and a third subcloning boosted 3D6-IgA expression from 4.6 ± 1.8 (3D6-IgA/5 C5) to more than 16 pcd (3D6-IgA/5 C5/13E9, 3D6-IgA/5 C5/13E9/11H7).

4B3-IgA transfectants initially produced between 0.04 ± 0.02 and 0.13 ± 0.07 pcd (Fig. 3B). Gene amplification and two subcloning steps increased IgA secretion from 0.11 ± 0.04 pcd (4B3-IgA/6H2) to 0.16 ± 0.04 (4B3-IgA/6H2/4 G4) and 0.17 ± 0.05 pcd (4B3-IgA/6H2/4 G4/5D1).

Western blotting was performed to analyze quality and isoforms of secreted IgA. Quality profiles of crude cell culture supernatants from 3D6-IgA cell lines at 0.1 μM MTX appeared heterogeneous (Fig. 4). All four clones produced IgA mono-, di-, tri- and tetramers at different ratios. Clone 3D6-IgA/8E10 expressed predominantly monomeric IgA while clones 3D6-IgA/3 G10, 3D6-IgA/5 C5 and 3D6-IgA/10D12 secreted mostly dimers. All four clones excessively secreted free light chains but no free IgA heavy chains were detected in the medium. As expected, J chain was present in IgA polymers, but not in monomers. Western blot analysis of 0.4 μM MTX adapted clones showed increased amounts of higher polymerized IgA isoforms (data not shown). Therefore, clone 3D6-IgA/5 C5 was selected for the production of culture supernatant for purification.

IgA isoform distribution was homogeneous among 4B3-IgA transfectants (Fig. 5). All four recombinant cell lines produced monomeric, but mainly dimeric IgA. A profound amount of free light chains was determined, but no free IgA heavy chains. J chain was detected in all 4B3-IgA polymers. Clone 4B3-IgA/6H2/4 G4/5D1 was selected for production of culture supernatant for purification based on IgA secretion.

3.2. Antibody purification by single step affinity chromatography

TFF was the initial purification step for removal of low molecular weight host cell proteins (HCP), IgA concentration (to 0.5 mg/ml) and adaptation of buffer conditions. Table 1

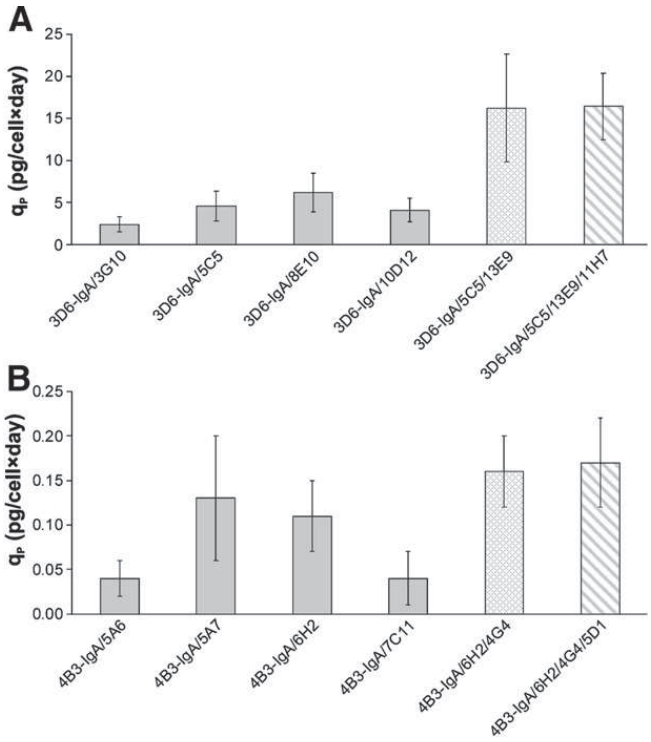

Fig. 3. Specific productivities of selected transfectants expressing (A) 3D6-IgA or (B) 4B3-IgA at various subcloning levels, where ▭ represents clones at 0.1 μM MTX, ▭ subclones at 0.4 μM MTX and ▭ the finally selected subclones at 0.4 μM MTX.

shows the high efficiency of this step with product losses of maximal 5%. VHH chromatography employing CaptureSelect human IgA eliminated substantial amounts of impurities from the load (Fig. 6) and very pure IgA fractions were isolated at high yields (Table 1). In total, 92% 3D6-IgA or 91% 4B3-IgA was recovered by this approach.

3.3. Antibody purification by multiple step chromatography

Immobilized jacalin chromatography could efficiently remove many impurities (Fig. 7) and eluates yielded 97% 3D6-IgA or 98% 4B3-IgA (Table 2). In a subsequent buffer exchange by TFF >95% of product was recovered without

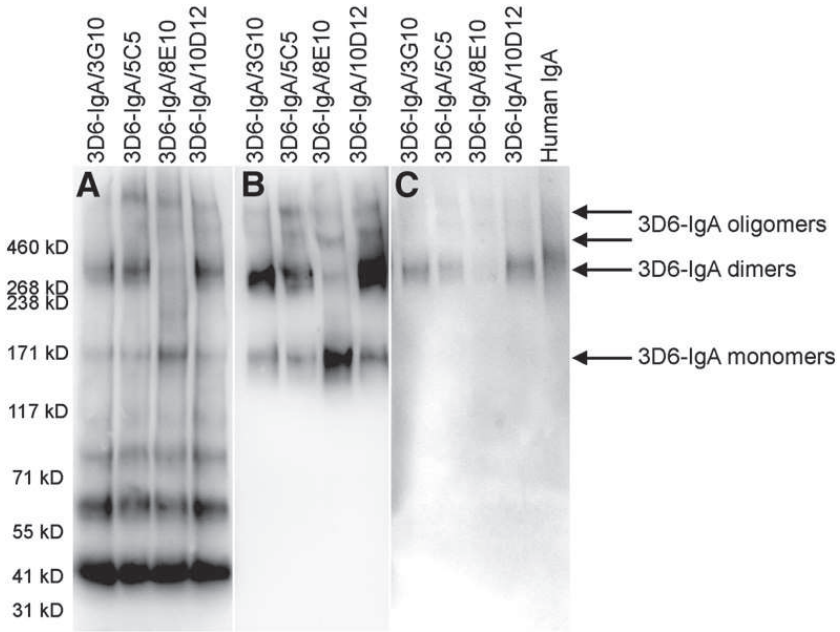

Fig. 4. Western blot of crude cell culture supernatants from 3D6-IgA expressing clones. Separation was conducted on NuPAGE Novex 3–8% Tris-Acetate gels applying a total of 260 ng 3D6-IgA of each clone. Product was labelled with an HRP-conjugated (A) anti-human κ-chain, or (B) anti-human α-chain specific antiserum. (C) J chain was detected with a rabbit anti-human J chain antiserum and an HRP-conjugated anti-rabbit IgG polyclonal serum. All immunoblots were developed via chemiluminescence.

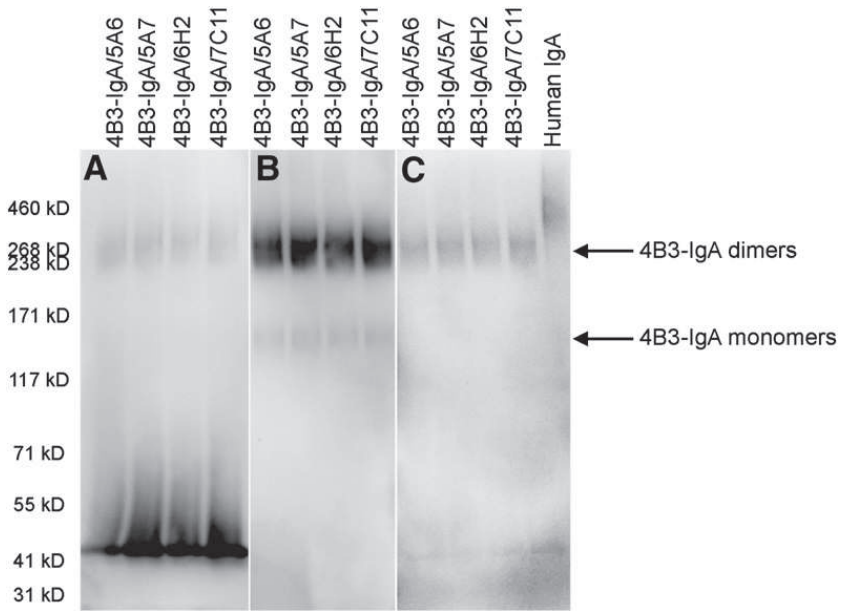

Fig. 5. Western blot of concentrated cell culture supernatants from 4B3-IgA expressing clones. 85 ng of each 4B3-IgA clone was separated and stained as shown in Fig. 4. Product was labelled with an HRP-conjugated (A) anti-human λ-chain, or (B) anti-human α-chain specific antiserum. (C) J chain was detected with a rabbit anti-human J chain antiserum and an HRP-conjugated anti-rabbit IgG polyclonal serum.

substantial mAb losses. After product polishing by AEX both IgAs were eluted yielding 87%. In a final HIC step, most remaining HCPs were removed and 3D6-IgA or 4B3-IgA yields ranged between 77 and 68%. HIC allowed the separation of dimeric 3D6-IgA from higher polymerized IgA. The total yield of this purification scheme was 61% for 3D6-IgA or 52% for 4B3-IgA.

4. Discussion

The recombinant expression of complex glycoproteins like IgAs is challenging as three polypeptides have to be assembled by the cell. We used the CHO/dhfr expression system (Alt et al., 1978; Kaufman, 1993) starting with a serum-free cultivated host cell line and generated cell lines which stably expressed 4B3-IgA and 3D6-IgA for several months.

The obtained specific productivities were similar or better than those reported by other groups which solely expressed IgA without J chain under serum-free conditions (Dechant et al., 2007; Beyer et al., 2009). Only Berdoz et al. reported higher values of up to 21 pcd dimeric IgA, but product could not be accumulated in the absence of FCS (Berdoz et al., 1999). Concerning the differences between 3D6-IgA and 4B3-IgA it should be mentioned that identical expression vectors and transfection methods, signal peptides and constant regions were used plus a panel of thousand initial transfectants were tested for each antibody but still clones produced 50-fold more 3D6-IgA than 4B3-IgA at 0.1 μM MTX and almost 100-fold more at 0.4 μM MTX. Cell biological investigations, quantification of gene copy numbers and mRNA will shed more light into these observations. Interestingly, higher polymerized or aggregated isoforms were detected for high producing 3D6-IgA clones but not for 4B3-IgA (Figs. 4 and 5).

For purification of cell culture supernatants we employed an anti-human alpha-chain VHH ligand for affinity chromatography. In our experiments, VHH chromatography enabled the isolation of both IgAs with >95% recovery and exceptionally high purities in a single step. Initial IgA elution with 0.1 M glycine at pH 3 yielded only 40% recovery (data not shown), but results were improved at pH 2. This pH was not considered to influence IgA stability as IgAs are naturally present in the gut at comparable pH values (McClelland et al., 1972). Product stability was additionally supported by electrophoretic analyses in which IgA bands appeared at the same positions before and after elution without any observable degradation. Also, the intact immunoreactivity of both IgAs to a recombinant HIV-1 trimeric glycoprotein (CN54 gp140) was confirmed by ELISA (data not shown). Taken together, much higher purities and yields were achieved by VHH chromatography compared to immobilized jacalin alone or the combined multiple step chromatography approach (Figs. 6 and 7) demonstrating the potency of VHH ligands for IgA purification. The described method may be adapted for large scale applications and the reduced number of steps optimizes process time and costs.

Table 1
Protein purification of 3D6-IgA and 4B3-IgA by single step chromatography. Obtained yields and purities for each individual step are shown.

	Yield (%)		Purity	
Step	3D6-IgA	4B3-IgA	3D6-IgA	4B3-IgA
TFF	95	95	-	–
VHH chromatography	97	96	++++	++++
Total	92	91		

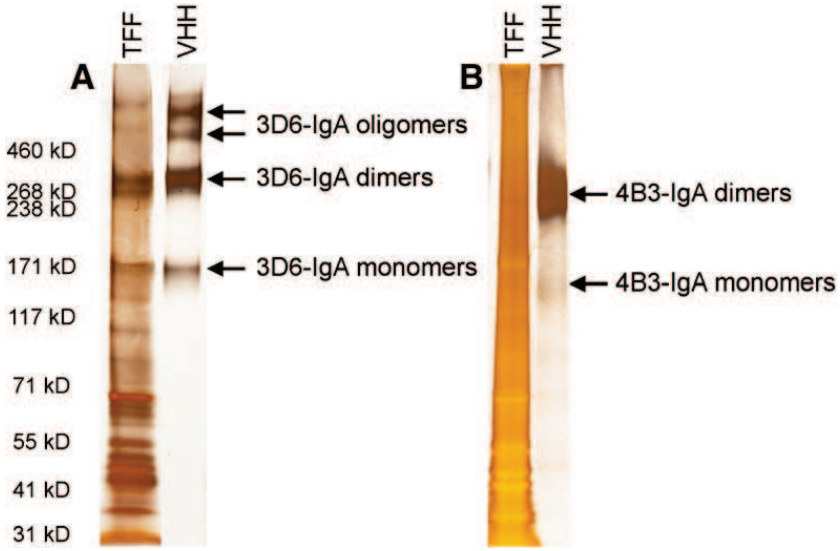

Fig. 6. Purity analysis of fractions obtained by single step chromatography. Equal amounts of (A) 3D6-IgA and (B) 4B3-IgA were separated by SDS-PAGE and visualized by silver staining.

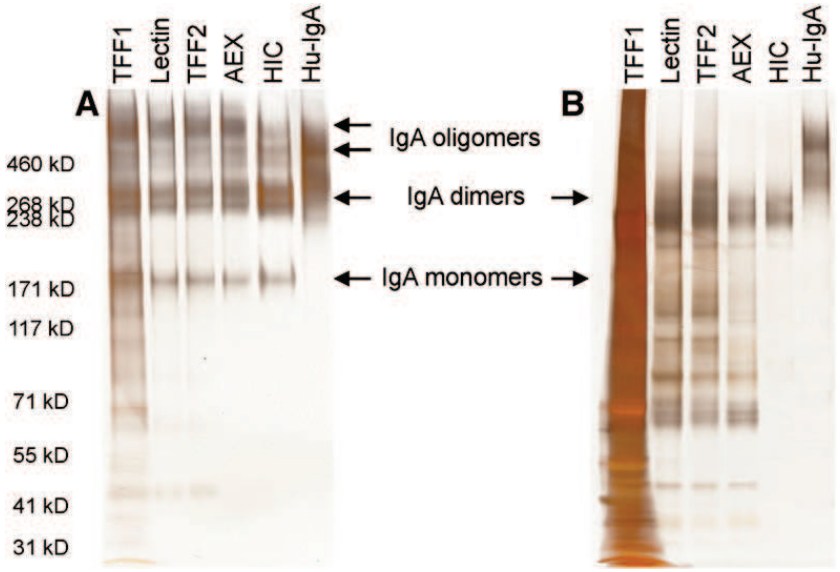

Fig. 7. Purity analysis of fractions obtained by multiple step chromatography. Equal amounts of (A) 3D6-IgA and (B) 4B3-IgA were separated by SDS-PAGE and visualized by silver staining.

In conclusion, we report the development of recombinant IgA production cell lines in the CHO system expressing J chain assembled IgAs. The employed alpha-chain specific VHH ligand permitted an efficient recovery of highly pure and functional IgAs in a single chromatographic step. The described method was superior to commonly applied IgA purification schemes and may be used as a generic capture step for the isolation of human IgA irrespective of their heavy and light chain subtypes.

Table 2
Protein purification of 3D6-IgA and 4B3-IgA by multiple step chromatography. Obtained yields and purities for each individual step are shown.

	Yield (%)		Purity	
Step	3D6-IgA	4B3-IgA	3D6-IgA	4B3-IgA
TFF	95	95	−	−−
Jacalin lectin	97	98	+	+
TFF	95	95	+	+
AEX	87	87	++	++
HIC	77	68	+++	+++
Total	61	52		

Disclosure statement

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Intracellular protein aggregation provokes low specific productivity in a recombinant IgA expressing CHO cell line

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Abstract

Background: The efficient production of recombinant proteins such as antibodies typically involves screening of an extravagant number of clones in order to finally select a stable and high producing cell line. Thereby, the underlying principles of a powerful protein machinery, but also potential expression limitations often remain unclear and are still poorly understood. To shed more light on this topic we looked at a previously generated recombinant cell line (4B3-IgA) which expresses rather low amounts of immunoglobulin A (IgA). The respective cell line was extensively characterized regarding growth rate and IgA productivity in long-term culture, gene copy number (GCN) and mRNA levels as well as immunofluorescence microscopy, flow cytometry and Western blotting of intra- and extracellular product. Results were aligned with the host cell line and another recombinant IgA producing cell line with better production properties from our lab.

Results: In the low producer 4B3-IgA higher GCNs, but mRNA levels similar to the well-producing clone could be determined. Flow cytometry of the low producer revealed an elevated signal for ER resident chaperones. Applying fluorescence microscopy large intracellular, granule-like spots, which appeared like protein deposits, were discovered. The identified spots consisted of mostly heavy chain molecules and originated from misassembled polypeptides. Furthermore, for the second clone, we identified IgA polymerization to structures larger than dimers. Polymerization started during or after having passed to the cell exterior since mono- and dimers were only intracellularly observed.

Conclusions: The clone 4B3-IgA demonstrated insufficiencies in protein folding/assembly identified by several intracellular high molecular weight products and therefore the observed low productivity was ascribed to the misassembled intracellular heavy chains. These accumulated protein deposits in turn evoked the expression of increased amounts of ER resident chaperones to combat the induced stress. Despite bottlenecks in protein processing the cells' quality check points remained intact and only correctly processed IgA was exported into the culture medium. The results of our study provide several important aspects of recombinant protein expression and revealed elevated chaperone formation in combination with limited antibody assembly. Our studies show that the nature of intracellularly expressed protein needs to be considered as a factor influencing a cell's productivity.

Keywords: Chinese hamster ovary (CHO), immunoglobulin A (IgA), cell line characterization, antibody production, antibody assembly, aggregation, ER chaperone

Background

Besides a few other mammalian cell lines, Chinese hamster ovary (CHO) cells remain the dominant choice as expression system for more than 70% of protein pharmaceuticals on the market [1]. Recombinant IgAs are not yet therapeutically available but progressively gain interest due to their unique role in mediating mucosal immunity. To meet the requirements of such complex proteins the respective production system needs to be properly investigated.

The efficient production of complex proteins typically involves the transcription of the relevant exogene(s), mRNA transport from the nucleus to the membrane of the endoplasmic reticulum (ER) for translation initiation and protein translocation into the ER, followed by N-glycosylation, folding and assembly of light chains (LC) with heavy chains (HC), and even J chain (JC) in case of polymeric IgA and IgM. Correctly processed molecules will be forwarded to the Golgi apparatus for further modifications and finally be secreted. Any of these steps is crucial and may be decisive whether a clone will become a high producing cell line or not.

High-level protein expression remains controversial and is often reported to coincide with high gene copy numbers (GCN) and mRNA levels [2, 3]. However, other reports claim that GCN does not necessarily correlate with antibody expression [4, 5] and clones with the highest mRNA levels are not always those with the highest productivity [6]. Mohan et al. (2008) has shown that an increased GCN or mRNA level was irrelevant for the secreted protein amount and therefore suggested that the bottleneck in enhancing productivity is a translational or post-translational process. Among the post-translational steps, the correct assembly of HC and LC has been proposed to be the major limiting factor for antibody production [7]. Incompletely assembled and unfolded HC is bound by BiP (Heavy Chain Binding Protein or GRP78) [8], recognized as an immature glycoprotein by the lectin chaperones calnexin (CNX) and calreticulin (CRT) and retained in the ER [9]. Polypeptides that cannot be refolded/assembled are removed from CNX/CRT by ER mannosidase I and ER degradation-enhancing mannosidase-like proteins and are eliminated by the ubiquitin-proteasome system by means of ER-associated degradation [10]. LC has a decisive role as its proper assembly with HC will trigger the release from BiP and thereby facilitates HC to fold correctly [11].

In the herein presented study we analyzed a previously generated recombinant cell line (4B3-IgA) which expresses rather low amounts of IgA [12]. We aimed to identify the underlying cause of the observed low productivity and received novel insights into antibody assembly and how cells cope with aggregation intermediates. The respective cell line was extensively characterized regarding growth rate, specific productivity, GCN

and mRNA levels, immunofluorescence microscopy, flow cytometry and Western blotting of intra- and extracellular product.

1. Results

1.1. Cell culture

The chosen culturing conditions of the finally selected cell lines favored viability (>97%) and growth for more than 20 passages. A decreasing μ from the host towards the recombinant cell lines was observed (figure 1A) and attributed to the inherent burden of heterologous protein expression. Notably, clone 4B3-IgA grew with a lower μ than clone 3D6-IgA, possibly indicating clonal differences in response to the recombination event. In addition clone 4B3-IgA had a low specific productivity of only 0.07±0.02 pcd. This was far less than commonly observed in our group (unpublished) and approximately 55-fold less than a normal IgA-expressing cell line such as 3D6-IgA (figure 1B).

1.2. Nucleic acids quantification

qPCR was performed in six technical replicates. The Cq values and calculated efficiencies were well reproducible (table 1). gDNA analysis revealed an overall higher exogenic GCN for the low producer 4B3-IgA than for 3D6-IgA (figure 2). On the genomic level clone 4B3-IgA contained two times more HC, three times more JC and four times more LC than the 3D6-IgA reference clone. Both clones incorporated more HC genes than JC than LC. This could be due to the presence of the dhfr amplification gene on the HC plasmid, whereas the neomycin resistance gene was located on the JC plasmid. No selection marker was included on the LC plasmid.

Despite higher gene copy numbers 4B3-IgA exhibited only half of HC and JC transcripts. LC was transcribed with the same range of efficiency and resulted in three times more LC mRNA copies. In contrast to gDNA results, LC mRNA content greatly exceeded that of HC and JC in both clones (figure 2).

1.3. Flow cytometry

Intracellular product content and potential ER stress signals were investigated by flow cytometry of recombinant and host cell lines. Importantly, all examined parameters were homogeneously allocated in each cell population. The intracellular LC signal of clone 4B3-IgA, relative to the λ-stained negative control, was lower than that of clone 3D6-IgA, relative to the κ-stained host cell line (figure 3A and B). HC polypeptides were slightly more abundant in the low producer 4B3-IgA (figure 3C).

Live-cell ER staining disclosed similarly intense signals for recombinant and host cell lines (figure 3D), indicating that their ER was of comparable size. Noteworthy differences

were found with an antibody specific for the KDEL sequence present on several ER chaperones such as BiP, PDI, GRP94 [13] and CRT [14]. The signal was stronger for the low producer (4B3-IgA) than for the reference clone 3D6-IgA than for the host cell line (figure 3E), adumbrating an increasing ER stress.

1.4. Immunofluorescence microscopy

Immunofluorescence microscopy confirmed results obtained from flow cytometry. DAPI staining of the nucleus and live-cell staining of the ER appeared similar in size, distribution and structure among the recombinant and host cell lines (data not shown).

The LC signal of clone 4B3-IgA was well-distributed throughout the cell and appeared rather faint. The HC seemed equally spread within the cell interior but in addition several large, granule-like HC spots could be visualized. In the overlay of LC and HC only a few spots appeared in white, indicating co-localized polypeptides (figure 4).

When we looked at the reference clone 3D6-IgA the LC/HC distribution was similar but the granule-like HC structures were absent. To investigate the assembly status of the antibody chains we immunoblotted intra- and extracellular product.

1.5. Immunoblotting of intracellular and secreted product

Anti-α chain staining of clone 4B3-IgA's intracellular content emerged as a broad smear distributed among several different molecular weights but most intense above the position of monomeric IgA (figure 5A). The respective band had a heterogeneous constitution since HC, LC and JC polypeptides were determined. Staining of the culture supernatant illustrated that only dimers were secreted, while the majority of the HC fraction remained secretion incompetent. This discovery further strengthened our hypothesis of incorrectly folded and/or misassembled polypeptides which were retained and hence accumulated in clone 4B3-IgA as observed by immunofluorescence microscopy.

The cellular lysate of clone 3D6-IgA contained only sharp bands of IgA mono- and dimers. Also tri- and tetrameric IgA was found but solely in the culture supernatant (figure 5B). This was an interesting finding since it indicated that IgA oligomerization started after product secretion. No intracellular bands suggested protein aggregation or assembly with chaperons.

Intra- and extracellular fractions of the low producer clone 4B3-IgA comprised an excess of unbound LC. Only little amounts of free LC were visualized in clone 3D6-IgA's cell lysate as well as in the supernatant. Free HC was not found in any fraction. As expected, all oligomeric molecules contained JC polypeptides in contrast to monomeric IgAs.

2. Discussion

Despite CHO cells are powerful hosts for recombinant protein expression the limits for poor secretion of distinct proteins remain often uncertain.

In this study a broad cell line characterization was performed with a low-producing, recombinant IgA expressing clone, 4B3-IgA. Results were aligned with data from another recombinant IgA producing cell line, 3D6-IgA, generated by identical methods but having better growth and production properties (figure 1).

qPCR experiments showed that mRNA levels between the two recombinant cell lines differed slightly, but were presumably not sufficient for the low specific productivity of clone 4B3-IgA (figure 2).

Intracellular LC and HC polypeptides in clone 4B3-IgA were well distributed, despite, several large granule-like spots which appeared as protein deposits were additionally observed for HC molecules (figure 4). Such cisternal dilations and the formation of so-called Russell bodies are known as a result of insufficiency of the folding and dislocation machinery under ER conditions which favor substrate aggregation within the lumen [15].

Staining of the endoplasmic reticulum revealed a comparable size for ERs of 4B3-IgA, 3D6-IgA and the host cell line (figure 3D), however, we found alternating amounts of proteins bearing a KDEL signal sequence (figure 3E) that is typically present on ER-retained chaperones such as BiP, PDI, GRP94 [13] and CRT [14].

Since unassembled HCs establish interactions with ER chaperones, we assumed increased ER activities [16] due stress signals induced by the accumulation of unfolded proteins [17]. Under such conditions, an inhibition of translation initiation can occur [3] which would additionally explain clone 4B3-IgA's reduced productivity. Folding/assembly were verified by immunoblotting of intra- and extracellular product. 4B3-IgA's cellular lysate contained a smear which was distributed from about 70 kDa to more than 460 kDa (figure 5) indicating for occasionally erroneous LC, HC and/or JC polypeptides assembly. However, although antibody assembly was prone to errors only correctly folded antibodies passed the cellular quality control and were secreted into the culture supernatant while wrongly processed polypeptides remained secretion incompetent. The obtained data represents an astonishing example of how a cell's endogeneous protein quality-check functions even for recombinantly expressed exogeneous molecules. Furthermore, when focussing on the "random intracellular assemblies" the strongest signal originated from the HC which may have been particularly difficult to process. These results were in agreement with our data derived from immunofluorescence microscopy where granule-like spots were observed only for HC and also flow cytometry indicated a high HC content in clone 4B3-IgA despite a lower productivity (figure 3C).

Anti-λ chain staining revealed that the majority of 4B3-IgA LC occurred as free intracellular entities which were secreted without being bound to HC (figure 5). Such an excess of free LC polypeptides in the ER was previously reported to be important for efficient protein folding/assembly processes [2] and was even proposed to correspond to higher secretion rates [18, 19]. However, in case of 4B3-IgA an excess of LC was found but the production rate was anyway low.

Another interesting finding, although not necessarily relevant for the productivity, was the observation that IgA was only found as mono- and dimer in clone 3D6-IgA. Oligomerization was induced either during or after product secretion (figure 5).

3. Conclusions

The underlying cause for clone 4B3-IgAs low productivity was ascribed to insufficiencies in folding/assembling of antibody polypeptides which the cell stored intracellularly as puncture protein deposits, supposedly so-called Russell bodies. Granule-like spots contained mostly HC but also other polypeptides which were randomly assembled into various high molecular weights. The intracellularly accumulated protein triggered a stress response as could be observed by an increased amount of ER resident chaperones. Remarkably, despite substantial difficulties in protein processing the cells' quality control remained intact and only correctly processed IgA was secreted. Summarizing, this study interlinks several important aspects of recombinant protein expression and demonstrates that limited antibody assembly can be combined with elevated chaperone formation. Furthermore, the nature of intracellularly expressed protein is often neglected during cell line development, but may greatly determine a cell's productivity.

4. Methods

4.1. Cell culture and determination of specific productivity

Recombinant CHO cell lines stably expressing the anti-HIV-1 antibodies 4B3-IgA and 3D6-IgA as human IgA1 were previously generated by transfection of HC, LC and JC vectors in equimolar ratios [12]. In this study, the 4B3-IgA expressing cell line was analyzed by several different techniques as described in the sections below. Results were aligned with data from the host cell line and another recombinant IgA producing cell line (3D6-IgA) with better production properties from our lab.

Host and recombinant cell lines were cultivated at 37°C in spinner vessels (Techne, UK) at 50 rpm in 50 ml medium. Every 3-4 days the total cell number was quantified using a Coulter Counter Z2 (Beckman Coulter, CA) and the cell viability was determined by trypan blue dye exclusion. Cell lines were split and re-seeded at 2.5×10^5 cells/ml. Recombinant cell lines were cultured for at least 20 passages to monitor growth rates (μ),

specific productivities (q_P) and stability of product expression. μ was calculated from the cell seeding concentration x_0 (cells/ml) and the finally achieved cell concentration x_1 (cells/ml) after time t (days) in culture prior to the next passage.

$$\mu = \frac{\ln(x_1 / x_0)}{t}$$

q_P was calculated in picogram antibody per cell and day (pcd) from the initial and final antibody titers T_0 and T_1 (μg/ml), respectively.

$$q_P = \frac{(T_1 - T_0) \times \mu \times 10^6}{x_1 - x_0}$$

Values for μ and q_P were averaged from all passages for each cell line. Samples for immunostaining and nucleic acid analysis were collected three days after passaging during the exponential growth phase.

4.2. ELISA

IgA in culture supernatants was quantified in a sandwich ELISA using a goat anti-human α-chain specific antiserum (Sigma, MO) for product capturing. Affinity purified 4B3-IgA or 3D6-IgA was prepared according to Reinhart et al. (2012), adjusted to 400 ng/ml and used as reference standards. 4B3-IgA and 3D6-IgA were detected with a peroxidase-conjugated goat anti-human κ-chain (Sigma, MO) or peroxidase-conjugated mouse anti-human λ-chain specific antiserum (Southern Biotech, AL), respectively. Product was stained with orthophenylenediamine and detected at a wavelength of 492 nm and a reference wavelength of 620 nm using an Infinite M1000 microplate reader (Tecan, Switzerland).

4.3. Preparation of genomic DNA and cDNA

Genomic DNA (gDNA) was isolated from 2 x 10^6 cells using the DNA Blood Mini Kit (Qiagen, Netherlands) according to the manufacturer's instructions. The concentration of isolated gDNA was quantified spectrophotometrically at an absorbance of 260 nm and the purity was determined measuring the ratio at 260 nm and 280 nm. gDNA samples were stored at 4°C.

Cellular RNA was isolated from 5 x 10^6 cells using Ambion Tri Reagent Solution (Life Technologies, CA) according to the manufacturer's instructions. To remove DNA contaminations from extracted RNA the preparation was digested with 3 U DNase I (Qiagen, Netherlands) for 30 min at room temperature together with 160 U RNase inhibitor (Life Technologies, CA) and then inactivated for 10 min at 75 °C before another

RNA precipitation step. Purified total RNA was dissolved in 25 µl RNase free water containing 60 U RNase inhibitor.

cDNA was obtained by reverse transcription. 1.5 µg RNA, 1µg random primers (Promega, WI) and 12.5 nmol dNTPs (New England Biolabs, MA) were incubated in a reaction volume of 14 µl for 5 min at 70 °C and 2 min at room temperature. Then, 40 U RNase inhibitor, 200 U M-MLV reverse transcriptase and buffer (both Promega, WI) were added to a reaction volume of 20 µl and incubated for 30 min at 37 °C before denaturation for 5 min at 95 °C.

4.4. Real-time PCR

Real-time PCR (qPCR) analysis was performed on a MiniOpticon qPCR device (Biorad, CA). Primers and the fluorogenic hydrolysis probes (table 2) were synthesized by Sigma (MO). Same primers and probes were used for the analysis of gDNA and cDNA. The reaction mix included iQ Supermix (Biorad, CA), 6 pmol primer and 4 pmol hydrolysis probe for HC, JC and ß-actin quantification or 12 pmol primer and 8 pmol hydrolysis probe for LC determination in 20 µl reaction volume. 3 ng pre-denatured (99 °C, 10 min) gDNA or 3 µL cDNA from a 1:50 dilution of the reverse transcription reaction was used directly for qPCR. Negative controls (NC), no template controls (NTC) and no reverse transcriptase controls (NRT) for transcript analysis were included in each run. The quantification cycle (Cq) was determined by linear regression and baseline subtraction using the CFX Manager (Biorad, CA). The mean qPCR efficiencies for HC, LC, JC and ß-actin were calculated from raw fluorescence data using the LinRegPCR software application, V12.17 [20-22]. Quantification was done by relative quantification with efficiency correction [23] using ß-actin as internal reference and expressed as ratios.

4.5. Flow cytometry

Cells were prepared for flow cytometry based on an established protocol [24]. Briefly, 1×10^6 cells were washed with D-PBS (PAA, Austria) and then fixed in 1 ml ethanol (70 %) for 20 min at 4°C. After washing twice in 1 ml Tris buffer (0.1 M Tris HCl, 2 mM $MgCl_2$, pH 7.4) cells were blocked with 20% FCS in Tris buffer for 30 min at 37°C. Cells were then incubated for 30 min at 37°C with 200 µl Tris/FCS containing an Alexa-Fluor-488-conjugated goat anti-human IgA antiserum (Dianova, Germany) and a biotinylated goat anti-human λ-chain (Novus Biologicals, CO) or κ-chain (Agrisera, Sweden) antiserum. Cells were washed and incubated in 200 µl Tris/FCS with a Alexa-Fluor-647-streptavidin conjugate (Life Technologies, CA) for 30 min at 37°C and re-suspended in 500 µl Tris buffer with 50 ng/ml DAPI (Sigma, MO) for analysis. ER-specific proteins were analyzed

using a DyLight-488-conjugated mouse anti-KDEL antiserum (Enzo Life Sciences, Austria).

Live-cell ER labeling was performed by incubating 1 × 10^6 non-fixed cells with 200 µl D-PBS containing 1 µM ER-Tracker Blue-White DPX (Life Technologies, CA) for 20 min at room temperature. Cells were washed, re-suspended in 500 µl D-PBS with 50 ng/ml DAPI and immediately analyzed.

In each experiment 10,000 cells were analyzed using a Gallios Flow Cytometer (Beckman Coulter, CA) at the wavelengths of 488 nm, 638 nm or 405 nm. The fluorescence emissions were measured with a 525/40 BP filter (Alexa-Fluor-488; DyLight-488), a 660/20 BP filter (Alexa-Fluor-647) or a 450/40 BP filter (DAPI; ER tracker Blue-White DPX). Non-producing CHO host cells were used as negative controls.

4.6. Immunofluorescence microscopy

Cells were prepared by ethanol fixation and stained using the same antibody conjugates and protocol as described for flow cytometry. 30 µl of the stained cell suspension was transferred onto microscope adhesion slides (Marienfeld-Superior, Germany) and incubated for 10 min. Cells were washed thrice with D-PBS and prepared for microscopy using the SlowFade Antifade Kit (Life Technologies, CA) according to the manufacturer's instructions. Four channel confocal images were taken with a Leica TCS SP5 II laser scanning microscope (Leica Microsystems, Germany) using sequential excitation at the wavelengths of 488 nm and 633 nm. Fluorescence of Alexa-Fluor-488 and Alexa-Fluor-647 was detected in the range of 500 nm - 552 nm and 690 nm - 750 nm, respectively. The microscope hardware setting was kept constant for imaging the wild type and the recombinant cells. Image processing was performed with Leica LAS AF and ImageJ software.

4.7. Immunoblotting of intracellular and secreted product

Intracellular protein was harvested by cell lysis using RIPA buffer (Sigma, MO) according to the manufacturer's instructions and the cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Switzerland) to avoid product degradation. Both the obtained extract and the secreted product were denatured in NuPAGE LDS 4× sample buffer (Life Technologies, CA), electrophoretically separated on NuPAGE Novex 3-8 % Tris-Acetate gels (Life Technologies, CA) and analyzed on Western blots applying the same antibodies as used in ELISA. J chain was visualized using a rabbit anti-human J chain antiserum (Antibodies-online, Germany) and a peroxidase-conjugated anti-rabbit IgG polyclonal serum (Sigma, MO).

List of abbreviations

CHO, Chinese hamster ovary

ER, endoplasmic reticulum

GCN, gene copy number

HC, heavy chain

IgA, immunoglobulin A

JC, joining chain

LC, light chain

qPCR, real-time polymerase chain reaction

Disclosure statement

The authors have declared no conflict of interest.

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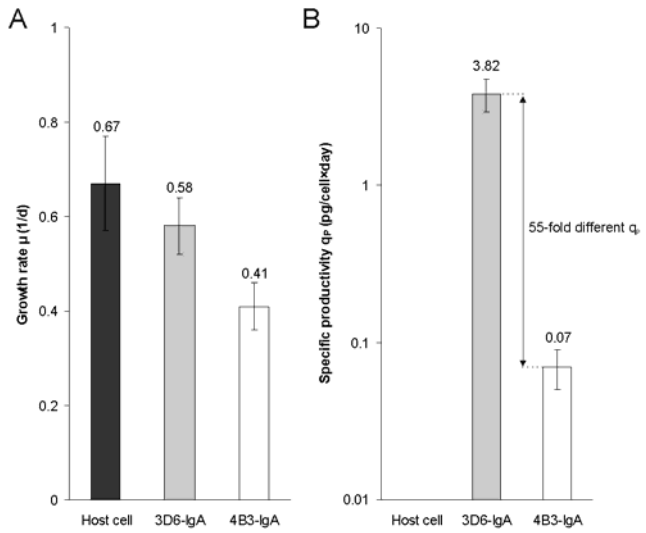

Figure 1: Growth and specific productivity of the investigated cell lines. (A) Growth rate and (B) specific productivities are shown for the host and recombinant cell lines and were averaged over all passages.

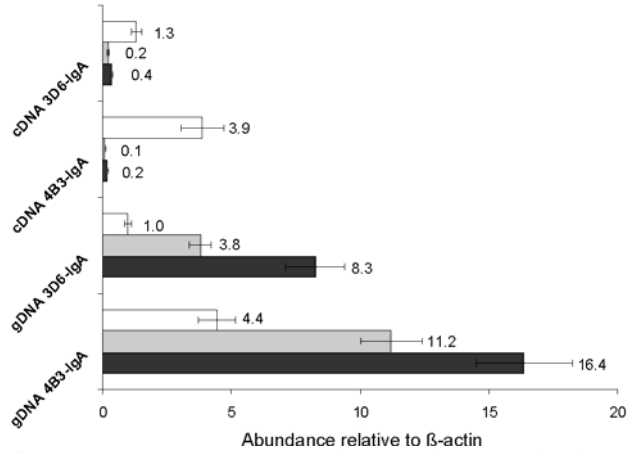

Figure 2: Gene copy number and transcript level of recombinant clones expressing 3D6-IgA or 4B3-IgA. The abundance of LC (□), JC (▒) and HC (■) genes was calculated relative to ß-actin.

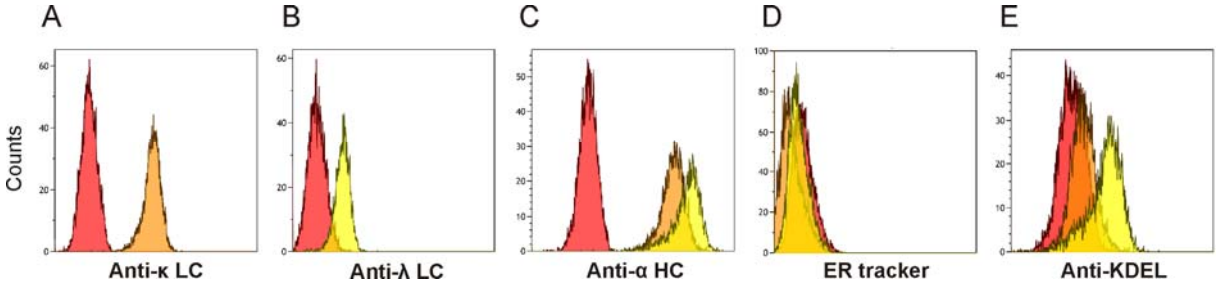

Figure 3: Cellular characterization by flow cytometry. The histograms show the recombinant clones 3D6-IgA (■) and 4B3-IgA (■) as well as CHO host cell line (■) after

staining with (A) anti-κ LC, (B) anti-λ LC, (C) anti-α HC, (D) ER tracker Blue/White DPX and (E) anti-KDEL reagents.

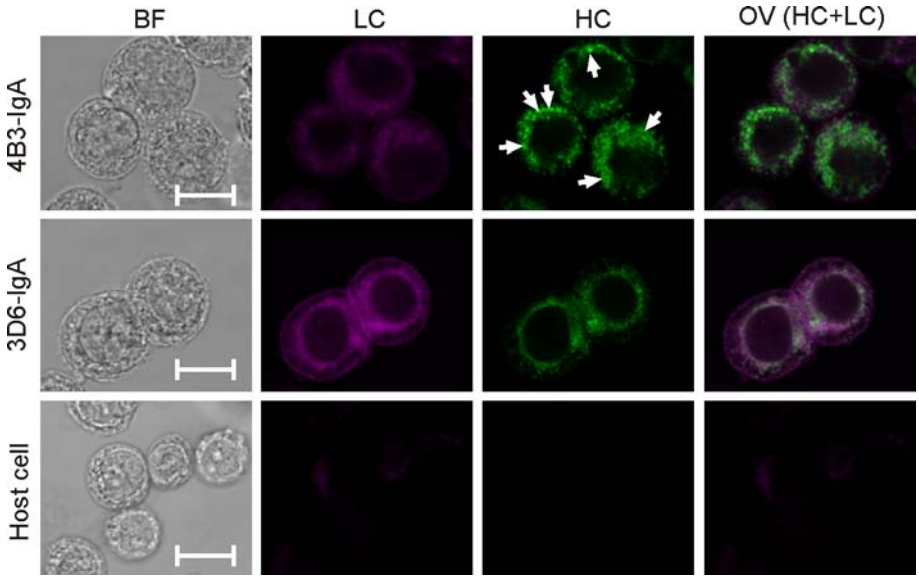

Figure 4: Immunofluorescence microscopy of cell lines. The recombinant clones 3D6-IgA and 4B3-IgA as well as CHO host cell line were bright field (BF) imaged. Intracellular LC and HC was detected and stained in purple and green, respectively. Co-localized LC and HC are shown in white color in the overlay (OV). Bar, 10 µm.

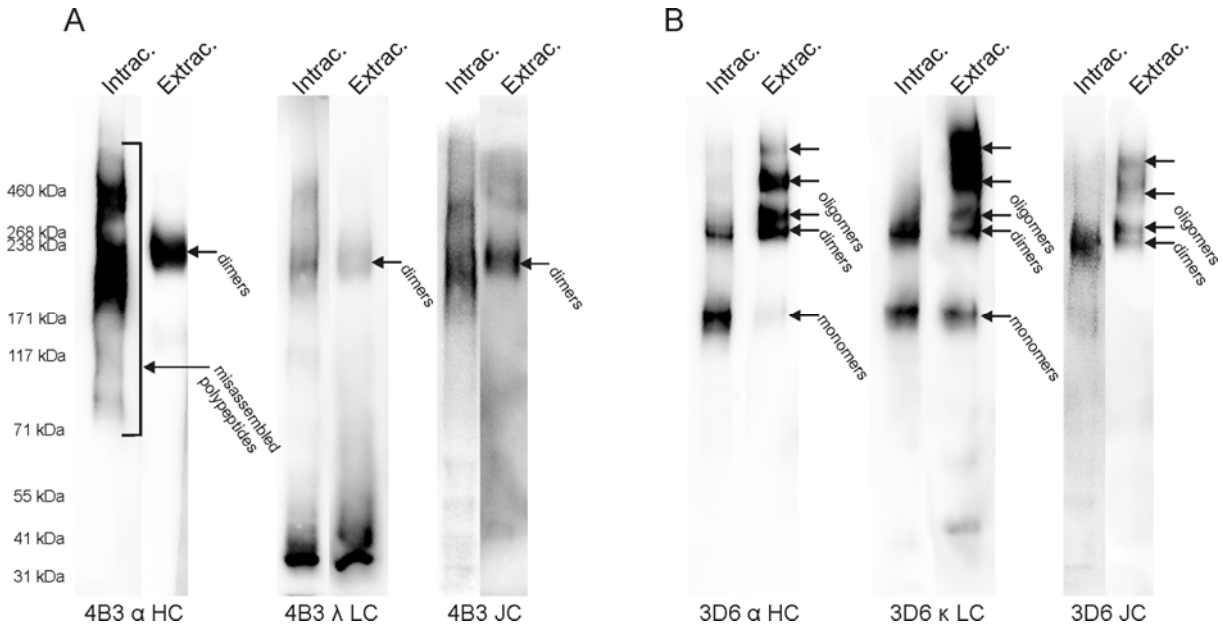

Figure 5: Immunoblot of intracellularly expressed polypeptides and secreted product. Protein isolates from the recombinant cell lines 3D6-IgA and 4B3-IgA were stained individually for their HC, LC and JC molecules.

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Table 1: Calculated efficiencies (E), Cq and ΔCq values and copies relative to ß-actin for gDNA and cDNA derived from clones 3D6-IgA and 4B3-IgA.

GOI	Target	Clone	Cq	max. SD [%]	E	SD (%)	ΔCq ß-actin	Copies relative to ß-actin
ß-actin	gDNA	3D6-IgA	24.60	0.20	2.07	2.22	n/a	n/a
		4B3-IgA	24.21	0.14	2.07	2.22	n/a	n/a
	cDNA	3D6-IgA	18.52	0.13	2.03	0.43	n/a	n/a
		4B3-IgA	16.25	0.63	2.04	1.33	n/a	n/a
HC	gDNA	3D6-IgA	23.56	0.16	1.95	3.32	-1.03	8.28
		4B3-IgA	22.11	0.14	1.95	3.32	-2.11	16.44
	cDNA	3D6-IgA	21.78	0.17	1.91	1.35	3.26	0.38
		4B3-IgA	19.50	0.68	1.97	1.53	3.25	0.20
JC	gDNA	3D6-IgA	24.81	0.03	1.95	0.94	0.22	3.80
		4B3-IgA	22.77	0.10	1.95	0.94	-1.44	11.20
	cDNA	3D6-IgA	24.52	0.23	1.82	0.87	5.97	0.22
		4B3-IgA	20.81	1.54	1.96	0.27	4.56	0.10
LC	gDNA	3D6-IgA	24.90	0.14	2.05	0.59	0.31	0.98
		4B3-IgA	21.50	0.21	2.11	1.21	-2.71	4.40
	cDNA	3D6-IgA	20.26	0.20	1.88	0.75	1.73	1.30
		4B3-IgA	15.02	2.36	1.98	1.30	-1.22	3.93

Table 2: List of hydrolysis probes, sense (s) and antisense (as) primers used for qPCR. Fluorogenic hydrolysis probes were 5'-labelled with 6-carboxyfluorescein (FAM) and 3'-labelled with the fluorescent quencher dye 6-carboxytetramethylrhodamin (TAM). All sequences are given in 5' to 3' direction.

Designation	Sequence (5'-3')	Amplicon size (bp)
HC s primer	AGTCCAAGACCCCTCTGACC	198
HC as primer	TACTTCTCCCGAGGCAGTTC	
HC probe	[6FAM]CCGGCAACACCTTCAGACCTGA[TAM]	
JC s primer	CTCTGAACAACCGGGAGAAC	193
JC as primer	GTTCCGGTCGTAGGTGTAGC	
JC probe	[6FAM]CACCTGTCCGACCTGTGCAAGAA[TAM]	
4B3 LC s primer	TCTGCCTGATCTCCGACTTC	185
4B3 LC as primer	CCTGGCAAGAGTAGGACCTG	
4B3 LC probe	[6FAM]CCCTTCCAAGCAGTCCAACAACAAG[TAM]	
3D6 LC s primer	TGTGCCTGCTGAACAACTTC	182
3D6 LC as primer	AGGCGTACACCTTGTGCTTC	
3D6 LC probe	[6FAM]AGCAGCACCCTGACCCTGTCCAA[TAM]	
ß-actin s primer	TGAGCGCAAGTACTCTGTG	78
ß-actin as primer	TTGCTGATCCACATCTCCTG	
ß-actin probe	[6FAM]CCATCCTGGCCTCACTGTCCACCT[TAM]	

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REVIEW Open Access

Rational design of HIV vaccines and microbicides: report of the EUROPRISE network annual conference 2010

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Abstract

Novel, exciting intervention strategies to prevent infection with HIV have been tested in the past year, and the field is rapidly evolving. EUROPRISE is a network of excellence sponsored by the European Commission and concerned with a wide range of activities including integrated developmental research on HIV vaccines and microbicides from discovery to early clinical trials. A central and timely theme of the network is the development of the unique concept of co-usage of vaccines and microbicides. This review, prepared by the PhD students of the network captures much of the research ongoing between the partners. The network is in its 5th year and involves over 50 institutions from 13 European countries together with 3 industrial partners; GSK, Novartis and Sanofi-Pasteur. EUROPRISE is involved in 31 separate world-wide trials of Vaccines and Microbicides including 6 in African countries (Tanzania, Mozambique, South Africa, Kenya, Malawi, Rwanda), and is directly supporting clinical trials including MABGEL, a gp140-hsp70 conjugate trial and HIVIS, vaccine trials in Europe and Africa.

Introduction

It seems clear that the EUROPRISE-sponsored studies reported herein are evolving within a dynamic HIV prevention landscape. Participants at the EUROPRISE Network Annual Conference discussed how EUROPRISE can best contribute to and facilitate the Global Enterprise Plan described by Alan Bernstein, executive director of the Global HIV vaccine Enterprise, and furthermore how promising data from the Thai RV-144 vaccine trial [1], the HIVIS vaccine trials [2], the Caprisa 004 tenofovir microbicide trial [3], and recent ART-PrEP (antiretrovirals for preexposure treatment) trials should influence our thinking and maximize research momentum. Such novel interventions should be considered along with more established prevention measures such as circumcision, condom use and diminishing transmission of HIV through the use of effective ART.

It was considered that novel prevention combinations are desirable and that members of the EUROPRISE consortium were particularly well placed to undertake studies investigating such combined effects. Possible combinations suggested were:

- The use of vaccines in circumcised men to further reduce transmission.
- The combined use of oral PrEP and microbicides to provide optimal systemic and localized drug loads.
- The combined use of vaccine candidates, microbicides and/or PrEP which may deliver improved protection and the following benefits even if suboptimal alone.
    - Providing protection during the immunization period.
    - Reducing infectious challenge.
    - Boosting local immunity (to HIV antigens).
    - Broadening localized resistance through protective immunity to other prevalent microbes.

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- Vaccine induced immunity covering intermittent non-compliance, break-through virus, and the prevention of evolution of drug-resistant virus.

A novel idea discussed during the meeting concerned the possibility that mucosal exposure to virus in the context of PrEP may lead to potentially useful local immune responses - such a phenomenon has been indicated in animals but is yet to be tested in man. Other animal studies have indicated that vaginal vaccination may induce mucosal immunity to HIV; this also should be tested in man. Similarly it is an intriguing possibility that vaccine induced immunity could be broadened through protected exposure to prevalent virus, or vaccine-microbicide combinations may provide better protection than either modality alone.

One expected result of even modest success in the field of HIV-1 prevention would be that the use of placebos in trials becomes unacceptable. However, together such prevention modalities may provide a pathway to lowering HIV incidence and to eventually reversing the epidemic.

This review reflects the EUROPRISE students' understanding of presentations at the EUROPRISE 4th annual conference. A detailed program of the meeting including abstracts of all presentations can be found at http://www.europrise.org.

Microbicides and novel antiviral compounds

Several novel studies of microbicides including clinical and preclinical studies were presented, and different aspects of microbicide research were addressed, including new microbicide candidates, combinations of reverse transcriptase inhibitors (RTIs) as potential microbicides, phase I clinical trials, and trials to test the acceptability of different formulations.

The increasing number of women infected with HIV in the sub-Saharan Africa pleads for the development of a protective tool against the virus that can be controlled by women. Microbicides have long promised to become such a tool. Many microbicide trials have failed to show any protection against the virus. However, results from the first successful clinical trial of a Tenofovir gel by the Centre for the AIDS Program of Research in South Africa (CAPRISA) [3], have given hope for the development of an effective product directed towards women.

The use of RTIs (reverse transcriptase inhibitors) as microbicides has been encouraged following the success of the CAPRISA trial. In this study, the overall protection against HIV infection was around 50% after the first year but protection was decreased to 39% after two and a half years [3]. A different approach, presented by Herrera et al, using a combination of an entry inhibitor and an RTI in cellular and colorectal explant models, provided evidence that targeting the virus at different steps of the viral replication cycle increases antiviral activity compared to drugs used alone, just like in the case of systemic infection.

Results from a phase I trial, led by Lacey et al, in which the safety and pharmacokinetics of a novel microbicide containing three anti-HIV-1 monoclonal antibodies (2F5, 4E10 and 2G12) were presented, showed that the formulation was safe and well tolerated. In addition, concentrations of antibodies sufficient to block retroviral transmission were maintained for many hours. These results suggest that the use of a combination of monoclonal antibodies, which could have an advantage over chemotherapy through their inability to generate antiretroviral drug resistance, should be further explored. The possibility of using entry inhibitors as microbicides has also been investigated and many proteins capable of blocking HIV infection by binding to the envelope glycoproteins have been identified [4]. One of these proteins - the bacterial protein azurin, which binds with high affinity to gp120 and therefore blocks HIV entry into host cells - was presented as a potential microbicide and/or a drug for the treatment of HIV/AIDS [5].

Research into inhibition of HIV entry into host cells is at an interesting stage due to the successful approval for clinical use of enfuvirtide, an HIV fusion inhibitor that binds to gp41. T-1249 is a second generation HIV fusion inhibitor and prevents entry of HIV into host cells and also has the ability to better bind to infected cells than enfuvirtide, making T-1249 an even stronger HIV fusion inhibitor. Inhibition of HIV entry may also be targeted through binding of single domain antibodies to conserved regions of the gp41 ectodomain, such as the HR1 region in gp41. Such synthetic antibodies could be a new approach in HIV therapy or even be used for HIV prevention in microbicides.

One microbicide candidate that can inactivate a wide range of HIV strains by binding irreversibly to gp120 is Cyanovirin-N (CV-N) [6,7]. In order to supply sufficient CV-N to cover the current at-risk populations, extremely large amounts of CV-N would need to be produced. Plants offer an inexpensive alternative pharmaceutical production platform to traditional systems. However, outdoor production of transgenic plants raises regulatory fears concerning product quality and uniformity. Hydroponic cultivation in greenhouses allows controlled production and the utilization of rhizosecretion. The latter could be advantageous for purification and harvesting time, provided that the production levels are sufficiently high.

In a project conducted by Luisa Madeira, a EUROPRISE PhD student, two hydroponic systems were evaluated for rhizosecretion of CV-N: a sterile in vitro

system and a non-sterile hydroponic system that was based on the Nutrient Film Technique. Manipulation of hydroponic medium by addition of plant growth regulators increased CV-N rhizosecretion considerably. Yields of up to 25 ug/ml per week have been achieved, raising the possibility that this system could be developed as a serious candidate for the scalable production of microbicides. Further optimization by manipulation of medium, light and temperature is being investigated.

For a microbicide to be effective, it is crucial that it is well accepted by women and therefore used frequently. In the CAPRISA trial, the correlation between the frequency of gel application and protection was assessed. It was shown that around 50% protection was achieved by high adherence, whereas for low adherence the long term infection rate was reduced to 39% [3]. In the same context, an acceptability study was carried out by Nel et al. and presented at the meeting by Luciana Maxim from the International Partnership for Microbicides (IPM). Three different formulations were tried by women in 3 different countries in Africa. It was shown that all 3 formulations were well-accepted, although there were preferred dosage formulations. The most preferred formulation, the soft-gel capsule, was associated with increased sexual pleasure. The presenters concluded therefore that availability of microbicides in multiple formulations may increase acceptability and/or adherence and thus increase effectiveness.

Preclinical and clinical HIV vaccine studies

A presentation dealing with HIV vaccine development introduced the 2010 strategic plan of the Global HIV Vaccine Enterprise focussing on ways to facilitate and accelerate the development of an HIV vaccine. A large number of potential clinical trials were discussed. However, only a few of the many possible trials have actually been conducted, and their extremely high cost and length makes a large increase in numbers of trials seem unlikely. Therefore we need to make better use of the few trials conducted, and in particular we should increasingly bridge basic science and clinical trials to get immediate feedback on how to optimize the design of antigens and vaccine protocols. A closer international collaboration between research groups as well as engagement of the industry, were suggested to be crucial [8].

The RV144 HIV vaccine trial is the only phase III vaccine trial that has shown a modest protection (31%) against HIV infection. It was conducted in Thailand where more than 16 000 participants received a viral vector prime and a protein boost [1]. A massive amount of data is now being analysed to identify possible correlates of protection. Vaccination did not affect viral load or CD4 counts in individuals who became infected, but further analysis will assess possible dissimilarities in immune responses observed between vaccinated and unvaccinated volunteers.

Results from prime-boost studies with the multigene/multisubtype HIVIS DNA and MVA-CMDR conducted in both Sweden and Tanzania were presented [2]. The combination is immunogenic and a low DNA vaccine dose administered intradermally is superior to a higher dose administered intramuscularly. Additionally an ongoing clinical trial addresses the effect of simultaneous electroporation on the effectiveness of the plasmid-based DNA prime. Recent results, including those from the RV144 trial, point at the potential utility of recombinant gp140 to further boost the DNA/MVA immunizations, and this will be integrated into an upcoming clinical trial. Particularly, innate and mucosal responses will be studied [9].

After the encouraging results of the RV144 vaccine trial in Thailand, HIV vaccine research has focused on the development of novel prime/boost vaccine strategies to further increase efficacy. Several posters presented innovative prime/boost strategies used in multiple combinations to find the best approach. Particularly interesting was the development of multigenic, multivector vaccines to fully optimize immunogenicity. Using DNA constructs with multiple HIV genes and boosting with different viral vectors, David Hallengärd, a EUROPRISE PhD student, could demonstrate an increased potency of the antibody response and more polyfunctional cytotoxic T-cells in a mouse model. The priming effect of HIV genes was previously shown both preclinically [10] and clinically [2]. To decrease the number of vaccinations needed to establish protection, an alternative scenario could include the use of a replication-competent modified foamy virus. The foamy viral vector could establish persistent infection and express the antigen without pathology creating long-lasting immunity.

For a prophylactic HIV-1 vaccine to be effective, the generation of protective immune responses needs to be localized at the site of viral entry, which in most cases is the mucosa. In many vaccine approaches, the HIV gp140 Env glycoprotein is used to generate antibody responses. However, the application of trimeric gp140 without adjuvant to mucosal surfaces did not elicit sufficient antibody responses. Katja Klein, a EUROPRISE PhD student, presented data from a study testing various adjuvants mucosally, in order to enhance mucosal antibody responses to vaccination. Briefly, the immunogenicity of Tetanus Toxoid (TT) and four different modified gp140 preparations were examined either alone, or in combination with polyethyleneimine, dimethyl-beta-cyclodextrin (DM-CD) or chitosan, as adjuvants to increase mucosal permeability of the antigens after intranasal, sublingual and intravaginal

administration in female BALB/c mice. Even though DM-CD has toxic properties, no negative side-effects such as local inflammation of tissue were observed in the study. The data demonstrated that all three permeation enhancers could increase antigen bioavailability after nasal, sublingual or vaginal application. Disappointingly, antibody responses after vaginal immunisation could only be achieved with the tetanus antigen and not with any of the gp140 formulations.

The PEDVAC trial presented by Paolo Palma, a EUROPRISE PhD student from the Ospedale Pediatrico Bambino Gesù, is the first paediatric study evaluating therapeutic vaccination with an HIV multiclade DNA vaccine in vertically HIV infected children. The children had stable CD4 counts and controlled viral load by antiretroviral treatment. The study enrolled 20 patients, aged 4 to 16 years old, who were randomized into two arms. The safety profile of the vaccine was absolutely satisfactory and no major side effects were reported in comparison to children not receiving the vaccine. Vaccination did not adversely affect the viral load or CD4 counts and preliminary cellular immunogenicity data showed reactivity to vaccine antigens. Evaluation of these results is in progress and may provide key information on the status and changes of antigen-specific immunity following DNA vaccination in HIV infected children [11].

A field of interest represented by several poster presentations increased our understanding of the mechanisms of action of the adjuvants used in combination with vaccines. Different posters showed that it is possible to modulate the immune responses in the human host. Noteworthy was the observation of Annette Sköld, a EUROPRISE PhD student, showing that the combination of two different TLR ligands such as CpG and poly I:C do not act in a synergistic manner but instead CpG inhibits poly I:C induced dendritic cell maturation. Another poster showed that polyethyleneimine used as a mucosal adjuvant is able to strongly polarize the type of T-cell response in a TH-2 manner. Moreover studies on chitosans showed that it is possible to use these molecules in vaccines to target specific cells to increase the effect of the vaccine. Thus different types of immune responses can be elicited using strategies of prime-boost vaccines, such as DNA and vectors or proteins, in association with these new adjuvants to obtain protection against different pathogens.

Animal models for vaccines

Protection from infection in animal models was discussed at various points during the meeting. Non Human Primate (NHP) models play a crucial role in HIV research, particularly in the development of HIV vaccines. However, it has recently been highlighted that these models should not be regarded as gatekeepers for the advancement of vaccine candidates into clinical trials [12]. This issue was addressed by Alan Bernstein with reference to the Enterprise strategic scientific plan for 2010 [8] which identifies two major roles for NHP research. Firstly, as a tool for furthering our understanding of the complex interactions between host and virus, especially at mucosal surfaces which are often difficult to sample in humans [13], and secondly to inform vaccine/microbicide candidate design and clinical trial strategies. Presentations at the meeting, summarized below, illustrate how these principles have been applied for many years within Europe and are currently being applied within the EUROPRISE network.

Work has been undertaken to characterize protection induced by live attenuated SIV in NHP. In 1992 it was reported that rhesus macaques (*Macaca mulatta*) vaccinated with a live attenuated SIV (Simian immunodeficiency virus), containing a deletion in the nef open reading frame (SIVΔNef), were completely protected from challenge with pathogenic SIVmac [14]. Since then several studies have resulted in protection or reduced viremia following challenge either systemically [15] or at the mucosae [16-18]. However, the mechanism of protection remains unclear. Although an attenuated virus will probably not be a suitable vaccine candidate in humans due to safety concerns, it remains a useful tool for elucidating both general correlates of protection and immune responses required to protect against HIV infection.

Martin Cranage had previously demonstrated in a study with SIVΔNef and SIVΔEnv (nef or env deletions), that the distribution of two different live attenuated SIVs was comparable to wild type virus infection, despite the inability of the SIVΔenv virus to replicate (17]. Macaques which received SIVΔNef were protected from challenge but the mechanism of protection was not defined. Indeed, although SIV-specific T cell responses were induced, they declined over time and following challenge an anamnestic response was not observed [19]. This study indicated that the replicative capacity of the virus was linked to the level of protection. This led to the question - is protection from superinfection due to the presence of the virus in target cells or do the replication kinetics allow maturation of the immune response? In order to address this issue, investigators used a virus where replication can be controlled as described below.

Martin Cranage and Neil Almond presented two macaque studies using SIVrtTA, a conditionally replication competent virus which has been manipulated so that its replication is controlled by the administration of an antibiotic (Doxycyline) [20,21]. The virus was able to replicate *in vivo* and kinetics were similar to SIVΔNef

except that the viral set point was lower. Following challenge with homologous virus, only limited protection was seen. However both SIVΔnef and SIVrtTA had an effect on circulating and mucosal T cell phenotype, and polyfunctionality was associated with replicative capacity. Building on this first study, Neil Almond presented data from a second study where SIVrtTA vaccinated cynomolgus macaques (*Macaca fascicularis*) received Doxycycline, allowing SIVrtTA to replicate before challenge with a heterologus wild type SIV. Almond's results showed that a 20 week infection period is required to achieve full protection against heterologus challenge. Additional data indicated that maturation of the macaque response against the virus was of key importance in conferring protection, and that this maturation continued after SIVrTA replication was halted. Whether the use of a different species of macaque contributes to the apparent superior protection against heterologus virus challenge is a point for consideration and may add to our understanding of how this vaccine works.

It is of interest to characterize mucosal immune responses in SIV infected macaques and to correlate these to long term survival. It has already been well described that there are a small number of HIV infected individuals who are able to control viral replication without medical intervention. Tina Schultheiss, from the German Primate Center, presented a cross sectional study characterising differences of cellular immune responses at several mucosal sites in SIV-infected rhesus monkeys comparing progressors with a high viral load (over 5×10^4 viral RNA copies/ml) and clinical signs of AIDS-like disease, and controllers with a viral load reduced from the peak of viremia to below 1×10^4 viral RNA copies/ml and clinically healthy during the study. As has been previously described, sampling one mucosal site is not indicative of immune response of the whole mucosa [22]. Therefore the lymphocytes in blood, bronchoalveolar lavage (BAL) and from duodenal and colonic biopsies were collected and characterised by flow cytometry. In addition, virus-specific immune responses were analysed using Gag-tetramers and intracellular cytokine staining. Results demonstrated that the functional virus specific immune response coupled with lower immune activation, as observed in virus-controlling animals, led to strong viral suppression both systemically and mucosally. This in turn resulted in the repopulation and maintenance of mucosal CD4+ T-cells and ultimately long-term survival, indicating that a successful vaccine candidate will have to elicit strong and long-lasting mucosal responses [23].

Oral vaccination is one of the most promising routes for inducing mucosal immune responses. However, studies presented at the meeting show that oral antigen delivery may induce tolerance. An effective oral vaccine should be able to avoid induction of antigen tolerance. Dominique Kaiserlain's presentation highlighted ways to break tolerance [24]. Her group has developed a mouse model to study the mechanisms of immune tolerance induction after oral antigen gavage. Results indicate that tolerance induction starts first in the liver and further continues in the gut and lymphoid organs of mice hyper-fed with antigen. In this study, liver plasmacytoid dendritic cells seemed to play a role in the induction of tolerance.

A deeper understanding of the properties of vaccine-induced antibodies, as well as the potential role of complement in eliciting immunity against HIV, will contribute to the design of an effective immunogen. In order to better understand the mechanisms of vaccine protection, the simian immunodeficiency virus (SIV) macaque model has been employed. Vaccination with attenuated SIV (SIVmacC8) was shown to result in sterilizing immunity against a subsequent wild-type viral challenge with SIVmac251 [25]. As was shown previously, uninfected 'cellular' vaccines and immunization with human leukocyte antigen (HLA) class I and class II proteins also resulted in protection of SIV challenged macaques. This outcome thus suggested a major contribution of antibodies specific for host cell proteins which are incorporated into virions during viral budding [26].

Indeed, the potential of HLA vaccines to protect against challenge with (SIV) [27] and HIV [28] has been previously demonstrated. In this case the protective status of challenged animals did not correlate with the presence of neutralization alone, but also a high reactivity of HLA-specific antibodies, thus emphasizing their importance in establishing immunity to HIV. Additionally, presence of complement was found to correlate with macaque protection from virus challenge and with neutralizing activity. These findings highlight the fact that future assays, which evaluate the potential of vaccine-induced immunization, should investigate multiple parameters, including complement and antibodies against HLA or other cellular components.

Studies in rhesus macaques immunized with recombinant HLA I and II, HIVgp140, SIVp27 and heat shock protein 70, linked to dextran backbones was reported to decrease the viral load and confer protection in 2 out of 8 macaques after intravenous challenge with SHIV carrying the corresponding HLA molecules. Correlates of protection included HLA-I-complement dependent neutralizing antibody activity. Serum transfer studies showed that antibodies from non-infected macaques were able to protect naive monkeys against subsequent rectal challenge. Alloimmunization of macaques using the MHC Mamu I and II alleles conferred similar protection. This makes the principle of immunization using proteins that are carried on the viral particles a promising target for further studies.

Effective primary antibody responses should be neutralizing

Broadly neutralising antibodies (Bnabs) mediate protection *in vitro* against a range of virus infections and thus it can be envisaged that humoral immunity, specifically neutralising antibodies, may play an important role in the protection against HIV infection or disease. The appropriate identification of neutralising antibodies during HIV infection or after immunization with vaccine candidates is therefore of utmost relevance. EUROPRISE has been actively involved in this issue through the NeutNet working group [29]. Recently, the research group of Fenyö at Lund University in Sweden developed a plaque reduction assay for measuring HIV and SIV neutralisation [30], which, however, demands manual dexterity and time consuming microscopic reading of the results. Enas Sheik-Khalil, a EUROPRISE PhD student in this group, presented the development of a high-throughput approach of this assay with a fast, objective automatic readout platform. The assay was implemented with an image analysis tool, which allows storage of the data and analysis of further parameters to gain deeper knowledge about the antibodies as well as the virus. The new assay has been applied to data sets collected within the framework of NeutNet phase II, which allows for a direct comparison with other HIV neutralisation assays as well as standardisation of the high-throughput plaque reduction assay, performed with both U87 CD4-cells and GHOST cells.

Although elicitation of Bnabs has been pursued in the development of vaccination strategies against HIV, no immunogen that can elicit a potent and broad neutralising antibody response has been developed so far. Indeed, this goal is hampered by the fact that the maturation of high affinity, neutralising antibodies to HIV envelope *in vivo* takes a long time, and the virus escapes neutralising antibody responses. Exciting and novel data concerning the viral characteristics in relation to the development of neutralising antibodies were presented.

One of the approaches, presented by Lara Mainetti, a EUROPRISE PhD student from the San Raffaele Scientific Institute, was to study the elicitation of neutralising antibodies to clonal viral variants obtained during acute infection and thereafter within 2 years, and determine their specific envelope-reactive properties relevant to formulation of an appropriate vaccine immunogen [31]. Neutralization sensitivity was investigated by testing a series of viral clones with consecutive serum samples obtained from the patients, as well as a panel of well described monoclonal antibodies including 2F5, 4E10 and 2G12. The autologous neutralisation sensitivity and the monoclonal antibody sensitivity patterns clearly underlined the specific evolution of each viral clone within and between patients. The clonal variation was further confirmed by the development of clonal variants able to differentially infect cells expressing CCR5 and/or CXCR4 chimeric receptors [32]. A detailed study of the development of the immunoglobulin classes against viral envelope monomers and trimers, and hundreds of peptides covering the whole envelope protein, showed differences in the viral targets of IgG and IgA as well as of responses to specific envelope epitopes. The antibody responses will be further analysed in relation to the clones' envelope sequences to highlight relevant immunogens.

Another current approach is to characterise epitopes of naturally occurring, very potent broadly neutralising, antibodies. These epitopes may then be used as immunogens to elicit HIV-1 specific neutralising antibodies with similar potency and breadth. Zelda Euler, a EUROPRISE PhD student, presented work on the comparison of early HIV-1 specific neutralising activity in five chronically infected patients from the Amsterdam cohort studies who developed Bnabs, including one elite neutraliser [33]. Clonal virus variants were isolated at multiple time-points covering the disease course from seroconversion until AIDS or death, and tested for sensitivity to autologous serum. The elite neutralizer developed Bnabs by 9.8 months post-seroconversion, in contrast to the other four patients who first developed their Bnabs at 30-35 months post-seroconversion. Viruses from later time-points had escaped autologous neutralising activity in all patients. Sera taken at regular intervals were tested against a panel of 6 heterologous viruses [34] and it was shown that the development of Bnabs coincided with autologus neutralising activity. In conclusion, the very early development of Bnabs in the elite neutraliser may suggest that the neutralising antibodies required less affinity maturation to become broadly neutralising as compared to antibodies from the other patients. A better understanding of such early Bnabs in the elite neutraliser could contribute to the design of new immunogens for an HIV-1 vaccine. Ideally a vaccine should elicit sterilising immunity against all or many different subtypes of HIV-1.

Marit van Gils, a EUROPRISE PhD student from the same research group in Amsterdam, presented a project, in which sera from 5 HIV^+ individuals who showed potent Bnabs as early as 2 years post-seroconversion, were characterised for their binding specificities to gp120 and gp41 [35]. The results showed that sera with broadly neutralising activity can contain antibodies against both gp120 and the MPER region of gp41, although the contribution of both specificities to such activity in these patients remains to be established. It is still unknown which specific epitopes are targeted by

the broadly neutralising antibodies from these patients. It might be possible that some of these antibodies are targeting unknown epitopes, on the other hand, multiple antibodies present at the same time could account for the breadth and potency of the sera. Future studies will further analyse other regions/epitopes of gp120 and gp41, as well as conformational epitopes/proteins, linear peptides and monomeric gp120.

Research supported by EUROPRISE has recently demonstrated that the HIV-1 envelope glycoprotein gp120 has evolved towards greater resistance to neutralisation over the 20 years of the epidemic [36]. Analyses were performed comparing neutralising sensitivity of isolated HIV-1 variants of the clonal subtype B from an Amsterdam cohort of infected individuals who seroconverted in the period between 1985 and 1989 (historical seroconverters) and another group of patients from Amsterdam who seroconverted between 2003 and 2006 (contemporary seroconverters). Detailed comparative studies showed that HIV-1 sensitivity to neutralization was significantly decreased in contemporary seroconverters. This was believed to be due to insertions of amino acids in the V1 region of gp120, as well as an increased number of N-linked glycosylation sites in this particular region of the viral envelope. These findings could explain why broadly neutralising antibodies, that can be found in a significant proportion of patients, do not change disease progression, as there seems to be a rapid selection of escaping HIV-1 variants [33]. Taken together, these results give crucial insight into host-virus interactions.

The discovery of multiple novel broadly neutralisation antibodies [37], including antibodies directed to the conserved CD4 binding site, was highlighted. Structural studies of the binding of these antibodies to gp160 are now inspiring the design of novel vaccine candidates. Particular aspects of the strategy have been the removal or masking of immunodominant and variable parts of the viral surface in order to direct the antibody response to conserved sites.

Cells behind antibody responses

The first papers reporting that antibody-dependent cellular cytotoxicity (ADCC) may play a beneficial role for the host during acute HIV infection date two decades ago [38,39]. At this time it was shown that antibodies mediate an antigen-specific attack by natural killer (NK) cell Fc receptors. Recently, the group of Christiane Moog has given a great input to this field. At this meeting the group presented a study in which ADCC with HIV-1-specific antibodies was performed using primary NK cells and autologous lymphocytes [40]. The autologous lymphocytes were stimulated with different HIV-1 strains and shown to give rise to HIV-1-specific ADCC activity, and the addition of HIV-1-specific antibodies increased the proportion of lysed cells. Studies to correlate phenotype of NK cells with ADCC activity are currently under way.

Antibodies are produced by B cells, which are affected during HIV infection and undergo extensive B-cell dysfunction due to hyperactivation and exhaustion of specific B-cell compartments. Data from Chiodi's group at the Karolinska Institute, presented by Nicolas Ruffin, a EUROPRISE PhD student, showed that B-cells from viremic patients have a higher expression of the IL-21 receptor on $CD27^+$ memory B-cells as compared to healthy controls, and that these cells display higher levels of the pro-apoptotic molecule Bim and lower levels of the anti-apoptotic molecule Bcl-2. Also, an inverse correlation between the levels of IL-21 receptor expression and the percentage of circulating $CD27^+$ memory B-cells suggests a possible role of IL-21 as an important cytokine involved in B-cell functions and differentiation during HIV-1. Therefore IL-21 could be used as a new target to prevent B-cell dysfunction in HIV.

During HIV infection several dysfunctions are found in the B cell compartment as shown in a poster presentation from Simone Pensieroso from the San Raffaele Scientific Institute. In fact all the B cell subpopulation frequencies including transitional, naive and activated memory B cells were altered in patients not treated with HAART. The application of successful antiretroviral therapy leads to normalization of percentages of cells from the B compartment but the subset of resting memory B cells, which are responsible for the maintenance of humoral immunity, is not restored even under HAART treatment [41]. As a consequence, antigen-specific humoral responses are lost in HIV-infected individuals. Indeed a less efficient response against the new pandemic influenza A (H1N1) vaccination was shown in HIV-infected patients both in a group of HAART treated patients and in a group of patients naive to therapy in comparison with healthy controls. Preserving memory B cell functions would allow a normal response against pathogens and a cross-neutralizing response against HIV.

Understanding the effects of HIV infection on the cells of the immune system would allow us to identify important targets for vaccine development, so we can preserve their functions. Several posters presented work emphasizing the role of dendritic cells (DC) and B-cells during HIV infection. For instance, deficiencies in plasmocytic DC function were among the earliest observations of immune dysfunction in HIV infection. However it was shown that HIV infection of these cells can be inhibited by neutralizing antibodies. The design of a vaccine inducing neutralization antibodies could prevent

pDC infection and preserve their role as vital link between innate and adaptive immunity.

HIV pathogenesis and endogenous targets for intervention

The genomes of primate lentiviruses have a significant bias in their nucleotide composition and their genetic code usage as compared with the genomes of their hosts. To evaluate the consequences this bias might have on the lentivirus-associated pathology, Nicolas Vabret, a EUROPRISE PhD student at the Pasteur Institute, compared the average nucleotide composition and genetic code usage of primate lentiviral genomes with those of their natural or experimental hosts, revealing that the more divergent the nucleotide composition of a virus is from its host, the more pathogenic it is. A similar correlation was observed by comparing the nucleotide composition of different HIV-1 subtypes (clade A, B, C, D & G) to that of the human genome. Subtype D was significantly more divergent than other subtypes, which is consistent with studies showing that subtype D infection is associated with a faster CD4+ cell decline when compared with other subtypes. To determine whether the sequence of the lentiviral genome itself could play a role in AIDS pathogenicity, the ability of a series of 500 bp long RNA fragments derived from the HIV-1 HxB2 sequence to induce type I interferon responses after in vitro transfection was analysed. Local divergence of HIV-1 RNA fragments strongly correlated with the ability to activate a type-I interferon response.

HIV-1 infects cells via interaction with CD4 and either CCR5 or CXCR4 as co-receptors, but only CCR5-using (R5) viruses are efficiently transmitted among individuals. CXCR4-using (X4) strains usually emerge during a late stage of infection. It has previously been demonstrated that CD4+ T cells from cord blood are permissive for R5 but not for X4 HIV-1 replication in vitro and that such a co-receptor dependent restriction occurs at a post-entry level [42,43]. Samanta Mariani, a EUROPRISE PhD student, recently investigated a different model of HIV-1 infection using expanded primary CD4 + T cells isolated from either healthy children or from children with congenital adenosine deaminase deficiency in severe immunodeficiency (ADA-SCID) [44], before and after gene therapy. As in cord blood cells, CD4+ T cells isolated from either healthy or ADA-SCID children confirmed the pattern described above. In contrast, CD4 + T cells isolated from healthy adult individuals supported both R5 and X4 virus replication equally. No significant differences were observed in terms of CD4, CCR5 and CXCR4 expression, or in the activation/proliferation state, of paediatric versus adult cells. Entry and reverse transcription of R5 and X4 HIV-1 in children's CD4+ cells were similar up to 72 h post-infection, while a steep increase of R5 HIV DNA accumulation was observed in cells infected with R5 but not X4 virus. This finding is strikingly similar to the observation of a post-entry block of X4 HIV-1 infection in cord blood derived CD4+ T cells. Identifying host correlates of permissive R5 and restricted X4 HIV-1 replication is clearly relevant, not only for a better understanding of HIV immunopathogenesis, but also for developing effective prevention strategies against HIV transmission.

As discussed above primary infection is most commonly accomplished by HIV-1 strains that use the CCR5 co-receptor (R5), while CXCR4 utilizing viruses (X4) emerge later, during chronic infection. Dendritic cell (DC) migration through an *in vitro* colonic epithelial transwell system was detected following incubation with R5 - but not X4 - viruses, suggesting that the ability of HIV to induce the elongation of DC cellular processes across the epithelial barrier is related to viral tropism [45]. The Env region was shown to be essential to triggering DC mobilization. Both R5 and X4 viruses, however, could be collected by subepithelial DCs via transcytosis, and transferred to $CD4^+$ T cells. Strategies to block this transmission could be relevant for the development of a combined antiviral and vaccine treatment.

There are also differences between R5 and X4 strains at a post-entry level. It has been shown that only R5 viruses replicate efficiently in cord blood CD4+ T cells. The transcriptional profile of CD4 cells at different time points after infection with isogenic R5 and X4 viruses was examined and approximately 900 and 1100 genes were induced by R5 and X4 envelopes respectively, while an additional 420 genes were mobilized by both viruses. Using bioinformatic tools, functional categories of genes differentially expressed in response to R5 versus X4 infection were identified [46]. The discovery of genes associated with the differential replication ability of R5 and X4 viruses could reveal new therapeutic targets for blocking viral spreading.

It is well know that host genetics can also affect the ability of HIV-1 to establish an infection. Recent findings suggest that human leukocyte antigen C (HLA-C) plays an important role in HIV-1 infection. Donato Zipeto from the University of Verona, showed that pseudoviruses produced from HLA-C silenced cells were significantly less infectious than those produced from non-silenced cells. HLA-C associated with gp120 was detected within CD4-CCR5-gp120 fusion complexes, indicating that a specific association between HLA-C and gp120 occurs in cells co-expressing the two proteins before the fusion process. HLA-C increased HIV-1 infectivity by interacting with Env glycoprotein. The interaction between fluorescently tagged HLA-C and Env molecules were studied using a bimolecular fluorescence complementation technique. Preliminary results

reveal a co-localization signal both in the endoplasmic reticulum and Golgi vesicles, suggesting an early association between the proteins. Studying the interaction between HLA-C and Env could reveal new targets for the induction of neutralizing antibodies as well as for the development of new compounds that, by interfering with this association, could control the virus.

Another factor that might be involved in the transmission and spread of HIV-1 is the level of T-cell activation enhanced by the WFDC1/ps20 protein of the Whey Acidic Protein family. On the one hand, ps20 has been shown to promote HIV infection in activated T cells by enhancing cell adhesion and viral transfer via a higher frequency of virological synapse formation on activated T cells with high expression of ps20. On the other hand, WFDC1 gene expression is suppressed in Th1 cells, and expression of ps20 negatively correlates with secretion of the effector cytokine IFN gamma. Blocking the HIV enhancing effects of ps20 might serve to limit virus spread.

One study presented during the meeting was based on the hypothesis that a useful vaccine against HIV-1 would rely largely on mucosal responses and on the role of T-cell priming for induction of a potent immune response [47]. Immunizations with antigens from SIV as well as with model antigens from *S. gordonii* and ovalbumin were used for intranasal immunizations in mice. Early activated T cells were found in the draining lymph nodes that expand and migrate to the distal sites. This was corroborated by the fact that locally (nasally) activated DCs themselves migrate to the draining lymph nodes.

Results were presented which shed light on protective immune responses from a different perspective, and which concerned the potential of non-mucosal immunization, and specifically studies of DCs activated by intramuscular immunization which home to the mucosa and drive mucosal responses [47]. Significant expansion of DCs positive for a mucosal homing marker ($\alpha4\beta$) was found after activation by intramuscular vaccination. Furthermore systemic DCs were found to efficiently induce the expansion of $\alpha4\beta$ T cells. These results suggest the potential for strong cross-talk between systemic immunization and mucosal responses. By investigating the mechanism underlying this cross-talk it was shown that systemic DCs have the ability to produce an inducer of $\alpha4\beta$ expression (retinoic acid) from a precursor, hinting at the possibility of enhancing mucosal responses by up-regulating enzyme cascades in conjunction with systemic vaccination.

Discussion groups on PrEP and animal models

Several focused discussion groups were held, two of which are summarized below. The groups were aimed at encouraging important collaborations and exchange of findings and ideas between researchers from international institutions and universities who work with similar models or techniques.

The first discussion group asked how we can assess ARV for prevention without placebo-controlled efficacy trials? With the results of the CAPRISA 004 trial, and the availability of safer and more effective anti-retroviral therapy (ARV), questions arise as to whether placebo-controlled trials will remain ethical and/or authorized in the future. EUROPRISE - comprising scientists, clinicians, and also community organisations involved in HIV preventative clinical trials - plans a methodology workshop addressing the assessment of new ARV candidates for HIV prevention in the absence of placebo-controlled efficacy trials. A review of the research road map for ARV prevention will be conducted, aiming at the identification of products already available and those in the pipeline. Major issues such as markers of efficacy for ARV prevention, new regulations, and safety will also be examined by the panel coordinated by Sheena McCormack, Medical Research Council, UK. EUROPRISE expertise on HIV research, from basic science to *in vivo* animal models, will be the basis for discussion, bearing in mind the crucial question: how ensure a drug, vaccine, microbicide or other preventative modality is preventing HIV infection in an efficacious manner by comparison to other efficacious treatment but no placebo.

An extremely exciting and possibly important route of HIV intervention is the prophylactic use of already established therapeutic drug regimens to protect HIV-naïve individuals from HIV-1 infection. One approach is the use of these therapies as pre-exposure prophylaxis (PrEP) which could be an additional tool for reducing the risk of HIV transmission. HIV-naïve individuals would take a single drug or a combination of drugs in order to reduce the risk of infection, once exposed to HIV. PrEP trials are being performed world-wide and EATG (European AIDS Treatment Group) collaborating with EUROPRISE is committed to bringing together researchers to further investigate the options of PrEP.

The second organised focus group discussed the use of animal models. Participants in this group came from institutions including the National Institute for Biological Standards and Control (NIBSC), the Biomedical Primate Research Centre (BPRC), the National Agency for AIDS Research (ANRS), the German Primate Centre, Istituto Superiore di Sanità (ISS), as well as universities including London, Innsbruck and Oxford.

The use of Non Human Primate (NHP) models still plays a crucial role in HIV vaccine development, and in the current environment of diminishing research spending, collaborations between NHP and other animal model investigators has become increasingly valuable. Although it has recently been debated just how much

the vaccine field should rely on NHP models, a general agreement remains that these animal models provide us with an important tool to study retroviral immune responses and protection. Results seen in these models hold at least some predictive value, as was seen with results from both the STEP trial and the Phase III VaxGen [48,49]. A major challenge for scientists trying to compare data generated at the different centres using NHP's across Europe is whether the assays used by each group are comparable in terms of sensitivity and specificity.

A major activity of this focus group has been to develop a range of materials that are shared by each centre that enable these questions to be addressed. For example 2 large pools of serum from cynomolgus macaques infected with SIVsmE660 and from rhesus macaques infected with SIVmac239 and 2 monoclonal antibodies from the NIBSC have been prepared for sharing in order to compare and evaluate SIV neutralisation methods. Furthermore, a batch of high titre SIV infection plasma diluted in uninfected macaque plasma will be made available by the NIBSC for distribution via the Centre for AIDS Reagents (CFAR) to evaluate assays that determine viral loads for SIV. To help standardize T-cell assays, a second round of lyophilised activated macaque T cell materials, suitable for both Intracellular Staining (ICS) and ELISPOT methods, will be available from early 2011. In addition the German Primate Centre offered to provide 100 vials of cryo-preserved Peripheral Blood Mononuclear Cells (PBMC) from a MamuA01+ Indian rhesus macaque infected with SIV and known to be responsive to Mamu A01 restricted epitopes for distribution via CFAR. In combination with peptides from CFAR and German Primate Centre protocols, the cells would provide a method for establishing anti SIV T cell assays for rhesus macaques and establish the impact of various parameters in the protocol.

Overall the NHP discussion group resulted in a successful boost to European collaboration and biological material exchange between participants.

Conclusions

The fourth EUROPRISE Network annual conference was held in an atmosphere of renewed optimism. Very many imaginative and novel strategies to be used in HIV intervention were presented and discussed and are described above.

More than 34 projects within the network are funded by the European Commission along with seven funded by the Gates Foundation and the NIH. Until now around 200 multi-author papers have been published in high impact journals, a weekly news bulletin and science update is provided and is available to non-EUROPRISE colleagues worldwide. The network is a major hub for providing AIDS reagents. The network is fully described at http://www.europrise.org.

An extremely successful facet of the network is the cadre of 65 EUROPRISE PhD students who are at the heart of the enterprise - the training scheme is recognized internationally and has been extended to students from China, India and Tanzania. 20 students who form a central part of the PhD School made individual presentations during the meeting. Topics covered included neutralising antibodies and neutralisation assays; microbicides and HIV-1 pathogenicity. As a part of their training the students have prepared this review of the fourth annual EUROPRISE conference in Lisbon in November 2010 with the theme 'development of EUROPRISE (and EU research) within a dynamic prevention landscape'. We include important comments from the students themselves concerning their views on the school: '*It is very important to provide a platform where students, post-docs and professors, as well as clinicians and industry representatives can meet, exchange ideas and share knowledge. The Europrise PhD school has helped me to broaden my scientific experience and knowledge. The school has given me the opportunity to meet scientists from different fields/interests, and the tasks and discussions during the courses gave me the opportunity to view my own research from different angles*'.

A priority for the EUROPRISE network in the coming year must be to secure funding for the continuation of this novel, productive, Eurocentric network. The EUROPRISE PhD school must continue. We will endeavour to continue integrated developmental research on HIV vaccines and microbicides, from discovery to early clinical trials, through excellent collaborative work set up in the past 4 years, some of which is described in this review. We feel that our emphasis on the co-usage of vaccines and microbicides is unique and may lead to some alleviation of the suffering which is still caused by HIV world-wide.

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Authors' contributions

All authors participated at the EUROPRISE conference as to be able to report on it. SB, KDC, MVG, DH, KK, LM, LM, PP, KR, DR, MR, NR, JS, KS, ESK, AS, HU, NV and SZ were in charge of the writing of dedicated chapters covering the different sessions of the conference. GS, RS, BW and FG organized the sessions and the writing, and corrected and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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MEETING REPORT Open Access

Rational design of HIV vaccines and microbicides: report of the EUROPRISE annual conference 2011

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Abstract

Europrise is a Network of Excellence supported by the European Commission within the 6th Framework programme from 2007 to 2012. The Network has involved over 50 institutions from 13 European countries together with 3 industrial partners and 6 African countries. The Network encompasses an integrated program of research, training, dissemination and advocacy within the field of HIV vaccines and microbicides. A central and timely theme of the Network is the development of the unique concept of co-usage of vaccines and microbicides. Training of PhD students has been a major task, and some of these post-graduate students have here summarized novel ideas emanating from presentations at the last annual Europrise meeting in Prague. The latest data and ideas concerning HIV vaccine and microbicide studies are included in this review; these studies are so recent that the majority have yet to be published. Data were presented and discussed concerning novel immunisation strategies; microbicides and PrEP (alone and in combination with vaccines); mucosal transmission of HIV/SIV; mucosal vaccination; novel adjuvants; neutralizing antibodies; innate immune responses; HIV/SIV pathogenesis and disease progression; new methods and reagents. These – necessarily overlapping topics - are comprehensively summarised by the Europrise students in the context of other recent exciting data.

Keywords: HIV, Vaccine, Microbicide, PrEP

Introduction

The 2011 Network Annual Conference of Europrise was held from 14th to 17th November in Prague, the Czech Republic, and was attended by 100 partners from 31 European institutions, two members of the advisory board, ten affiliated partners and eight invited guests. Scientific data from earlier meetings were summarized previously [1,2]. New important data from oral and poster presentations were discussed by all collaborators at the conference and are summarised here by our PhD students. All participants at the meeting were greatly encouraged and excited by positive data from recent microbicide and vaccine trials, in the context of which the meeting's eight sessions dealt with the latest European clinical trials of pre-exposure prophylaxis (PrEP) with vaccines, microbicides and drugs in both man and animal models, using conventional and novel strategies, and aimed at inducing measureable innate and specific immune responses, both cellular and humoral.

One of the outstanding features of the Europrise Network of Excellence has been a work package dedicated to a PhD student training programme. In this context, young researchers have made exchange visits between laboratories of the Network and have attended dedicated workshops and conferences. During Europrise's lifetime, 35/64 students from 30 laboratories have completed their postgraduate studies, 20 of these students were directly funded by the Europrise programme.

HIV vaccines

Several strategies to hasten the development of an effective HIV vaccine were presented. These strategies included the production of novel vector platforms, the identification of potent adjuvants, the development of delivery devices and novel challenge models. A description of these novel immunization strategies is given, and their implications are discussed.

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Clinical trials

Immunological correlates of protection

The RV144 Phase III trial is the only trial in which the clinical efficacy, estimated at 31%, of an HIV vaccine has been demonstrated in man [3]. This prime-boost strategy was an attempt to combine an avipox recombinant vector expressing human immunodeficiency virus type 1 (HIV-1) Gag, Pro, with a bivalent gp120 protein boost (AIDSVAX B/E) [3]. This trial involved 16,400 Thai subjects. The modest efficacy signal in the RV144 trial has enabled a formal search for correlates of risk (increased or decreased risk of infection within the vaccinated cohort), involving a pre-specified plan of six primary and a large number of exploratory analyses. Analyses compare immune responses in vaccinated infected persons with those in vaccinated, uninfected persons [4,5]. Jerome Kim from the Walter Reed Army Institute of Research presented data showing that 2 of the 6 pre-specified primary analyses, corrected for multiple comparisons, were identified as correlates of risk that may be used as hypotheses for future testing. The first was a measure of the magnitude and breadth of anti-HIV Env IgA binding that was directly correlated with infection (RR 1.54; $p = 0.027$), although no enhancement of infection was seen compared to the placebo groups. The second was an indirect correlation between IgG binding to the V1V2 loop of gp120 and a 43% reduction in the HIV infection rate (RR 0.57 $p = 0.015$). Sieve analyses of breakthrough viruses, as well as antibodies from vaccinated uninfected volunteers, suggest that antibodies against the V2 loop may be associated with escape mutations in breakthrough viruses. Taken together, these findings support the original modest efficacy of the RV144 trial, and suggest hypotheses that can be tested in future animal challenge experiments [4].

Scientists from IDIBAPS (Institut d´Investigacions Biomèdiques August Pi in Sunyer and University of Barcelona) reported the results of the phase I clinical trial RisVac02 [6]. The study's purpose was to evaluate the safety and immunogenicity of a modified vaccinia virus Ankara (MVA) vector expressing monomeric gp120 and the fused Gag-Pol-Nef polyprotein of clade B (MVA-B). Thirty HIV-1 negative volunteers were given three intramuscular injections of MVA-B or placebo at weeks 0, 4 and 16 and followed for one year. The vaccine was safe and well tolerated: a number of adverse events were reported but they either were not related to the vaccination or were injection site reactions. The majority of the individuals (75 %) exhibited broad and polyfunctional CD8+ and CD4 + T-cell responses, especially directed against Env. Responses were durable, being detectable during the entire follow-up period, and were mainly found within the effector memory T-cell subset. Antibody responses to Env were induced in almost all the vaccinees and HIV neutralizing activity against BX08, tier 1, was observed in 33% of the volunteers [7]. Taken together, these findings suggest MVA-B as a promising HIV-1 vaccine candidate. Further studies are needed to assess the boosting effects of recombinant proteins. These results reflect the wider recognition that pox-based vectors represent promising components of potential HIV vaccines, the basis for the formation of the Pox Protein Public Private Partnership (P5, by Sanofi and Novartis, see AVAC.org) initiated with the objective of building on these and other important results to develop further pox-protein vaccines [8].

Another Europrise study (HIVIS) was reported which has advanced prophylactic vaccination by priming with multigene DNA, followed by MVA-CMDR of the type CRF A_E; thereafter the intention is to boost with the Env glycoprotein subtype C, the novel concept of prime-boost-boost. Consecutive development of cellular and then antibody responses to many HIV-1 subtypes was described [1,2,9]. A serological study to determine the breadth and magnitude of antibody responses to inserts in DNA, viral vector or protein components of the HIVIS vaccine is underway, supported by an innovation grant from Europrise.

Susan Barnett from Novartis described the planned development within the context of the Pox Protein Public Private Partnership (P5) of a proof-of-concept trial in South Africa, building on the findings of the RV144 trial but using clade C inserts and protein (gp120). The aim is to develop new envelope proteins and reagents for the subtype C HIV-1 epidemic. A pox virus (ALVAC) prime developed by Sanofi-Pasteur will contain the highly immunogenic ZM96 gp120–TM [10] and the boost will contain two subtype C gp120 proteins (TV-1 and 1086 C) formulated with the licensed potent vaccine adjuvant MF59. Use of gp120 monomers instead of trimers has been prioritised in order to make comparisons with data from the RV144 trial [3].

Data were presented on a novel T-cell immunogen, termed HIVconsv, designed by Tomas Hanke and Andrew McMichael (University of Oxford) to address HIV-1 diversity. Fourteen of the most conserved regions of Gag, Pol, Vif and Env have been assembled into one synthetic DNA construct. To ensure coverage of the virus diversity, each consensus fragment was derived from different HIV-1 clades [11]. The sequence coding for the HIVconsv immunogen has been inserted into plasmid DNA, MVA and into non-replicating adenovirus of chimpanzee origin ChAdV-63 [12]. The three candidate vaccines administered in combination were highly immunogenic in mice and macaques. A phase I/IIA clinical trial (HIV-CORE002) is currently underway to evaluate their safety and immunogenicity in healthy adults. Preliminary results suggest that the HIVconsv-based vaccines are safe and able to induce T-cell responses.

Studies of attenuated viruses and virus-like particles

In order to be useful as immunogens, inactivated virions should preserve their immunogenicity and be safe for the

patient. Plana and collaborators (Hospital Clinic-IDIBAPS, Barcelona) investigated the immunogenicity of a reverse transcriptase (RT) defective virion (NL4-3/ΔRT). Using stored lymphocytes from asymptomatic HIV-positive patients, they showed that NL4-3/ΔRT viruses induced T-cell responses in vitro in 56% of the individuals tested, while only 24% of them were responsive to the NL4-3 wild type virions inactivated with aldrithiol-2 [13]. The immune responses were significantly stronger against the RT-defective viral particles and included both CD4+ and CD8+ restricted responses. The results encourage further exploration of NL4-3/ΔRT viruses as additional reagents for screening HIV-1 specific responses in HIV seropositive individuals and vaccinees.

Further interrogation of the mechanisms of protection provided by attenuated viruses was reported in non-human primate (NHP) studies. Martin Cranage presented work performed by Maria Manoussaka as part of a collaboration with Neil Almond's team at NIBSC and Ben Berkhout's team at AMC, demonstrating that infection of macaques with SIVmac239Δnef or a doxycycline (dox)-dependent conditional replication variant of SIVmac239Δnef, designated SIVrtTA, increases the proportion of circulating T effector memory (Tem) (CD28-CD95 + CCR7-) cells with a concomitant reduction of T central memory (Tcm) (CD28 + CD95 + CCR7+) CD4+ and CD8+ cells only under replication-permissive conditions. Similar changes following infection were found in gut mucosal-homing (α4 + β7+ and β7+) T-cells but the changes were also maintained after doxycycline withdrawal. Also, global phenotype data of polyfunctionality of SIV-specific T-cells in the periphery and in the gut of the animals were presented. These data may contribute to our understanding of the mechanisms of resistance to superinfection.

The mechanisms behind protection induced by live attenuated SIV are still unclear. One of the theories is that the failure to superinfect 'immunized' monkeys is due to competition for available target cells rather than immune control. To uncouple replication from immune response, Oliver Hohn (Robert Koch Institute) produced a 181 bp nef-deletion version of Klaus Überla's SIVmac RT-SHIV that expresses the HIV reverse transcriptase. Unlike wild-type SIVmac, this virus was highly susceptible to the antiviral compound nevirapine. Eight rhesus macaques were infected with nef RT-SHIV; after allowing the immune response to develop for 40 weeks, four were given a daily dose of nevirapine (together with four 'unvaccinated' control animals). At 48 weeks, all 16 monkeys (vaccinated and unvaccinated, with and without nevirapine treatment) were challenged with wild-type SIVmac239. During the vaccination phase, all animals developed virus specific B- and T-cells. Peak viral load in vaccinated macaques after challenge was 2-3 orders lower than in control animals, and by week 10 all vaccinated animals were virus negative. Most importantly, there was no evidence that suppressing virus replication by nevirapine diminished the ability of the immunized macaques to resist infection by the challenge virus. These exciting results led to the conclusion that the suppression of virus replication in vivo was not the result of competition for infectable targeT-cells through active replication of the vaccine virus.

An alternative approach to attenuated viruses is the development of virus-like particles (VLP). This proved to be successful for the group of Buonaguro et al (Natl. Cancer Institute Pascale, Naples) which used the insect baculovirus system to produce VLP. These VLP vaccines, which are non-infectious and safe, show promise with or without HIV DNA priming [14].

Preclinical challenge models

Anti-viral CD8+ T-cell responses are commonly studied mechanisms, thought to play a protective role during HIV-1 disease progression. Several presentations at the Europrise meeting focused on novel T-cell vaccines in NHP models, with a low-dose repeated rectal challenge. Such models may give us better insight into the potential clinical utility of vaccines following a physiologically relevant challenge.

Neil Almond from NIBSC presented data on a novel vaccine designed to promote potent T-cell responses to multiple epitopes, based on a strategy designed and developed by Steve Patterson (Imperial College). Mauritian cynomolgus macaques were immunized intra-dermally with recombinant DNA and boosted with a recombinant non-replicating Adenovirus expressing wild type SIV full length Gag, with an ubiquitin sequence fused to the amino terminus, or a fragmented SIV Gag gene with an ubiquitin sequence fused to the amino terminus of each fragment. Animals were challenged repeatedly intra-rectally with low-dose SIVmac251. No difference in protection was seen between the three groups of vaccinated animals receiving the fragmented or full length SIV Gag. However, there was evidence that vaccinated macaques were less likely to acquire infection compared with naive challenge controls, though the effect did not quite achieve significance at the 5% level. These data suggest that modification of SIV Gag based antigens by either ubiquitin fusion strategies or fragmentation provide no significant advantage to to a vaccine that provided mildly beneficial effect. Additional studies are underway to compare the immunogenicity of these different vaccines as well as alternative routes of vaccination.

In a further study, reported by Christiane Stahl-Hennig from the German Primate Center, rhesus macaques were subcutaneously primed with single-cycle immunodeficiency virus (SCIV), followed by either an oral spray application or an intramuscular injection of either a recombinant Adenovirus (rAd) expressing SIV Gag/Pol and Env, or a recombinant fowlpox virus (rFPV) expressing SIV Gag and Env. Animals were then repeatedly challenged intrarectally

with low-dose SIVmac251. The vaccination regimen with immunization with oral rFPV followed by the systemic rAd was superior to the reverse strategy, as shown by the delayed acquisition of highly pathogenic SIV and reduced viral load post infection.

Klaus Uberla (University of Bochum) presented a strategy designed to direct antigens to dendritic cells (DC) and enhance T-cell antigen presentation, using a novel modality where Gag p27 is fused with a single-chain antibody that is specific for the DC-restricted antigen uptake receptor, DEC205. Macaques received the vaccine intramuscularly and were later boosted with virus-like particles to induce a humoral immune response. Somewhat counter-intuitive results showed that animals vaccinated with control antibodies coupled to Gag p27 made stronger IFNγ CD4 and CD8 T-cell responses than animals vaccinated with p27 fused to the DEC205 antibodies. After repeated low-dose rectal challenges there was a trend to faster infection in the group with the higher IFNγ responses and animals with a high IFNγ response were infected significantly faster than poor responders. These findings raise questions as to whether vaccine-induced immune responses may under some circumstances lead to increased risk of acquiring SIV infection.

The role of APOBEC 3 G (apolipoprotein B mRNA editing cytokine deaminase) and AID (activation induced deaminase), innately expressed by B-cells, in protection against SHIV infection was discussed by Thomas Lehner (GKT, London). Macaques were immunized with recombinant MHC constructs linked to HIV/SIV antigens, HSP70 and dextran. Results showed significant upregulation of AID in B-cells and upregulation of APOBEC 3G in CD27+ memory B-cells and CD4+ effector memory T-cells in immunized animals. Following challenge with SHIV SF162.P4 viral load was inversely correlated with AID in B-cells and APOBEC 3 G in B- and T-cells of infected animals, suggesting that the MHC/SHIV vaccine construct stimulated parallel responses by the two deaminases that might be involved in pre-entry and post-entry control of SHIV replication [15].

In a SHIV-based model, presented by Mark Page (NIBSC), macaques were vaccinated with an HIV-1 W61D rgp120 envelope construct and challenged intravenously with heterologous viruses. Neutralizing antibodies were detected against rgp120 homologous to the vaccine, but only limited neutralization was seen against the two heterologous SHIV challenge strains. Protection against infection correlated with T-cell proliferative responses specific for peptides present in HIV-1 W61D rgp120. However, transfer of serum from the vaccinated animals with measureable titres of neutralizing antibodies to naive animals did not confer protection, highlighting a putative important role of CD4+ T-cells in protection from HIV-1 infection.

Measles vaccine vectors have been reported as a novel strategy with a potential to deliver heterologous antigens into the antigen-processing pathways, promoting strong CD4+ and CD8 + T-cell responses [16]. In this context, Richard Stebbings (NIBSC) reported an immunogenicity study to characterize cellular immune responses induced by a recombinant measles vector carrying inserts for HIV-1 Clade C Gag, RT and Nef (MV1-F4) in cynomolgus macaques. A significant increase in the T-cell responses to the HIV insert was detected in animals immunized with both low and high dose MV1-F4. Of interest, the addition of a second boost further increased CD4+ T-cell responses in both groups despite the presence of high titres of measles neutralising antibodies, although this was not the case for CD8+ T-cell responses.

Novel immunization strategies

A major hurdle in the development of HIV-1 vaccines is the construction of immunogens capable of inducing antibodies that can broadly neutralize HIV-1. In order to prevent initial infection of host cells or limit early events of viral dissemination, neutralizing antibodies must target the surface envelope glycoproteins of many human immunodeficiency viruses, which are highly variable in sequence and structure [17]. Indeed, as principal viral target of neutralizing antibodies, the HIV-1 viral spike has evolved to evade antibody-mediated neutralization. Anna-Lena Spetz (Karolinska Institutet) presented data on a new vaccination strategy under development to overcome the high diversity of HIV-1 by inducing immune responses towards relatively conserved regions of gp120 and gp41. Since the variable epitopes of Env are generally more immunogenic than the conserved parts, immune responses are preferentially induced to variable regions. In order to focus B-cell responses to conserved neutralizing epitopes of Env, Spetz et al. applied a heterologous prime-boost strategy consisting of a DNA prime comprising a mix of three HIV-1 Env clade B gp140 constructs, followed by a boosting with a very different Env trimeric gp140 protein, administered to rabbits. They hypothesized that by mixing three different envelopes during the DNA prime, the concentration of antigen derived from the conserved regions would be higher than that from the more variable regions. Boosting with a different Env protein as compared to the DNA prime would further increase the likelihood of targeting constant regions of Env. The results showed that high titres of heterologous HIV-1 neutralizing antibodies were induced against Tier 1 viruses in the TZM-Bl assay and against Tier 2 viruses in the A3R5.7 assay. These data support further exploration of such heterologous regimens for induction of broadly neutralizing antibodies.

Previous work performed by Andrea Cara at the Instituto Superiore di Sanità has shown that integrase defective lentiviral vectors (IDLV) expressing HIV-1 gp120 can be used for the induction of polyfunctional CD8+ T-cell responses

and gp120-specific serum antibodies in mice [18]. In addition, *ex vivo* transduction of human macrophages and DC with IDLV expressing the influenza matrix protein M1 induced expansion of antigen-specific polyfunctional CD8+ T-cells. At the EUROPRISE meeting, Cara presented new data demonstrating that the addition of the SIV VpX protein to IDLV vectors enhances transduction efficiency in human and simian DC, thereby improving antigen presentation. Recent studies have demonstrated that host cell SAMHD1 acts as an HIV-1 restriction factor by inhibiting viral DNA synthesis [19]. The SIV VpX protein targets molecules in SAMHD1 to the proteasome for degradation, thereby enabling viral replication. Thus, by inhibiting SAMHD1-mediated restriction of viral replication, the SIV VpX protein may enhance the transduction efficiency of IDLV in human and simian DCs, thereby admitting the use of lower doses of vaccine, which improves the safety profiles for IDLV. Other studies of retroviral vector use were performed by Pepe Alcami et al. from Instituto de Salud Carlos III, Madrid. They demonstrated that RT-defective virions acted as effective immunogens for T-cells.

Rico Blochmann, a young investigator from the Robert Koch Institute, described the development of novel replicating foamy virus vectors capable of prolonged antigen presentation. Infection with low-level replicating retroviral foamy virus is often symptom-free, and the viral particle could be used as an alternative vector in an HIV vaccine construct. A foamy hybrid virus has already been tested in hamsters, with rapid responses to encoded proteins, and a vector for rhesus macaques has been developed. Two reading frames of the viral genome were successfully removed without affecting viral replication in the macaque model. A viral vector expressing GFP and a variety of HIV CTL epitopes have been developed and are being tested *in vitro*.

A new strategy for genetic HIV vaccination based on heterologous prime-boost regimes using different vectors and viral genes was presented by Marc Reudelsterz from the Robert Koch Institute. This approach, designed to avoid a decrease in vaccine efficacy following repeated vaccination using the same vector, was adopted in parallel for HIV- and SIV vaccines. Codon-optimised constructs (*rev-nef-tat-gag* and *pol*) were used to prepare vaccine vectors based on plasmid DNA, recombinant adeno-associated virus (rAAV) and recombinant adenovirus 5 (rAd5). The magnitude and breadth of T-cell responses were increased following heterologous triple-immunization of mice with DNA-, rAd- and rAAV-based immunogens, especially against the Pol and Gag gene products of HIV and SIV.

An innovative method for HIV vaccine delivery was presented by the EUROPRISE student Charlotte Pollard from the Institute of Tropical Medicine in Belgium. She has delivered Gag mRNA complexed to DOTAP/DOPE, a cationic lipid used for transfection subcutaneously in mice to be processed and presented by immune cells (unpublished). In an *in vivo* killing assay, Gag-expressing splenocytes were shown to be selectively killed. When combined with a Gag mRNA - p24 protein boost, both IgG1 and IgG2 antibodies were detected, as well as Th1 and Th2 responses. In addition, inflammatory DCs were detected in lymph nodes one day after vaccination. Other means to enhance transfection consist of electroporation of DNA- or RNA-based antigens significantly raises immunogenicity of the expressed protein [20].

Microbicides & Pre-exposure prophylaxis (PrEP)

Clinical studies

Development of an effective microbicide against HIV-1 continues to be a major target for the HIV community. The microbicide session of the Europrise meeting gave a comprehensive update on preclinical and clinical studies of novel potential microbicides.

Ian McGowan (University of Pittsburgh) discussed microbicide development in the context of the encouraging CAPRISA 004 microbicide efficacy trial [21] and provided a comprehensive review of the NIH-funded Microbicide Trials Network's clinical trial portfolio (see Figure 1).

Results from the CAPRISA 004 trial of tenofovir 1% gel and other trials of oral pre-exposure prophylaxis (PrEP) of tenofovir support the topical and oral use of antiretrovirals for prevention of HIV infection. Differences in protection rates across these clinical trials may be explained by a variety of factors, including the heterogeneity of study populations, the route and frequency of dosing, as well as differential levels of tenofovir metabolites in the vagina compared to the rectum after oral dosing, and much higher levels of active drug in the genital tract after topical antiretroviral treatment. Although the precise threshold of drug required for protection has yet to be determined, it was shown that topical administration achieved the highest levels of drug in the genital tract and low systemic levels.

A major driver of overall effectiveness of either microbicides or oral PrEP was adherence; this should be taken into account in the development of future trials and microbicide delivery strategies. Besides improving the methods for collecting information on product use, priority should now be given to establishing the safety of tenofovir gel in pregnancy, adolescence, and rectal use.

Tenofovir as pre-exposure prophylaxis (PrEP) has been shown to significantly reduce HIV incidence, alone or in combination with emtricitabine. Whereas the CAPRISA microbicide trial observed a 39% reduction in HIV acquisition with a 'before and after' sex schedule of tenofovir vaginal gel [21] and the iPREX PrEP trial observed a 44% reduction with daily oral Truvada (tenofovir/emtricitabine), the FEM-PrEP trial showed no effect for Truvada in women [22]. In May 2011, several researchers

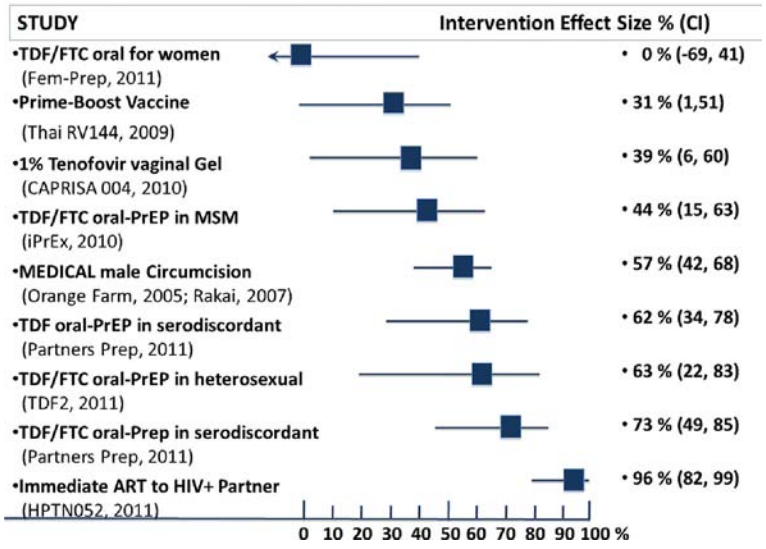

Figure 1 Reduction of HIV transmission by selected biological or drug interventions. Modified from Shattock et al [34] and expanded by us.

came together in a EUROPRISE workshop to better understand how adherence and biology explained the diverse effectiveness in these three trials. The most likely biological hypothesis was that when drug was administered orally, the level of active drug in the cervico-vaginal tissue was influenced by endogenous and exogenous female hormones. This question is now being investigated in a clinical trial. The promise of antiretroviral-based prevention also inspired a satellite meeting co-organised by AVAC, EATG and the Forum for Collaborative HIV Research during the 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention (July 2011 in Rome). This meeting concluded that it was time for the development of a multi-stakeholder consensus statement on the need to expand efforts to use antiretroviral-based combination prevention to achieve community-level control of HIV infection. Today we have far more options for preventing HIV than when EUROPRISE started (Figure 1) but considerably more research will be required before we know how best to deploy these alone and in combination in public health programmes.

New components for a microbicide

Neutralizing antibodies have been considered as novel microbicide candidates, since antibodies directed against conserved regions of HIV-1 proteins may inhibit HIV entry into host cells. Llama heavy-chain antibody fragments (VHH) are a class of molecules recently described as potent cross-clade HIV-1 entry inhibitors. Laura McCoy et al. (University College of London) showed that VHH antibodies isolated from libraries derived from llamas immunized with recombinant gp120 or trimeric gp140, have neutralizing activity against several strains of HIV-1. In particular, one potential candidate neutralizing antibody, J3, was identified on the basis of its capacity to neutralize the majority of HIV-1 pseudoviruses tested. Moreover, the importance of llama antibodies was underlined by their characteristic of being chemically and thermally stable and by their low production cost. Feasibility studies are now needed to determine the developmental pathways needed to bring this approach to clinical trials.

The EU funded Pharma-Planta Integrated Project was presented by Julian Ma (St George´s, University of London) as a potential platform for large-scale production of anti-HIV neutralizing antibodies produced in transgenic tobacco plants. He stressed the fact that this approach represents a new affordable manufacturing platform for proteins and microbicide combinations. Indeed, following cGMP approval for production of antibodies in plants, a clinical trial application has been approved, allowing a Phase I clinical trial to start. The results of the trial are anticipated to be available in late 2012.

Reverse transcriptase (RT) inhibitors might also form important components of an effective microbicide, as demonstrated by the CAPRISA 004 trial of tenofovir gel. TMC-120, a Non-nucleoside Reverse Transcriptase Inhibitor (NNRTI), is currently being evaluated in a phase III efficacy trial, while other NNRTIs are being tested in preclinical trials. Susan Fetherston et al. (Queen's University, Belfast) presented data on MC1220, another NNRTI evaluated in a non-human primate challenge study. MC1220 has previously been demonstrated to have potent anti-HIV activity in vitro and partial efficacy when formulated as a liposomal gel in macaques exposed to RT-SHIV [23]. Dr Fetherston presented results on the use of MC1220-

loaded vaginal rings in macaques and showed the presence of detectable drug levels in both vaginal fluid and plasma during the entire period of ring-placement. Drug release from vaginal rings was also demonstrated in *in vitro* experiments using simulated vaginal fluid. After the PK study (days 0-28), the same macaques were included in a challenge study. A one-week washout period was followed by placement of either a placebo (n = 4) or a 400 mg MC1220 ring (n = 6) on Day 35. Macaques were subsequently challenged with vaginally-administered RT-SHIV162P3 once a week for four weeks, on days 42, 49, 56 and 63. In the placebo arm, 1 of 4 macaques was infected within two weeks of placebo ring placement and following a single vaginal challenge; the remaining three macaques in the placebo arm were infected within four weeks of ring placement and following three challenges. The active ring provided a greater degree of protection, with one macaque infected by week 4, another by week 5, and a further two by week 6. At the end of six weeks, and following 4 challenges, 2/6 macaques remained uninfected. These results make MC1220 a promising compound for further development as a topical microbicide, and vaginal rings a promising device for delivering microbicides.

The ultimate goal for prevention is to increase the efficacy of individual strategies, providing a comprehensive blockade to HIV infection. Studies are underway to determine whether the potential protection delivered by microbicides could be increased by combining them with vaccination. Such a concept is at the heart of the Europrise Network of Excellence. Roger Le Grand et al. (CEA, Paris) presented preliminary data on a study performed in Cynomologus macaques where animals were immunized intranasally with trimeric gp140 subtype B and C proteins, followed by two intramuscular boosting immunizations with the same two proteins formulated in MF59. Animals were then challenged intravaginally in the presence or absence of 1% Tenofovir microbicide gel. The immunization schedule induced good systemic and vaginal immunity as measured by specific IgA and IgG levels, and presence of neutralizing antibody to tier 1 viruses in the serum but not in the vaginal secretions. The combination of vaccine with microbicide showed enhanced protection against SHIV challenge when compared to animals treated with microbicide alone; both microbicide alone and the combination provided significant protection against low-dose repeat viral challenge when compared to naïve challenged animals. Ongoing studies will assess the efficacy of the vaccine alone.

Mucosa and HIV-1/SIV transmission

Mucosal biology and viral infection

The potential influence of the human vaginal microenvironment on HIV transmission was addressed by Jordan Kyongo et al. (Institute of Tropical Medicine, Antwerp, Belgium). The presence of soluble immunomodulatory factors was evaluated in the genital fluids of a cohort of healthy Caucasian women. It was shown that levels of IL-1α, IL-1ra, IP-10, MIP-1b and the antimicrobial protein β-defensin fluctuated during different phases of the menstrual cycle. Such fluctuation may modulate susceptibility to HIV infection, potentially an important consideration when designing vaginal intervention strategies.

In contrast, Kevin Mendonca, a student from the University of Siena, demonstrated the importance of the seminal compound spermine in down-regulating TLR expression on vaginal epithelia cells. He raised the possibility that as activation of some TLRs, for example TLR-3, significantly reduces viral infection, the action of spermine might be a factor that influences male-to-female transmission of HIV through dampening of innate antiviral responses. However, the *in vivo* relevance of these findings requires further investigation.

Mariangela Cavarelli from San Raffaele Scientific Institute in Milan discussed studies concerning the susceptibility of dendritic cells and CD4+ T lymphocytes to HIV-1 infection using an *in vitro* model of intestinal mucosa. The influence of epithelial cells on HIV-1 infection of DCs and CD4+ T-cells was investigated to provide a better understanding of virus transmission events at the intestinal mucosa. Supernatants from the epithelial cell line Caco-2 were used to condition monocyte-derived DCs and CD4+ T-cells, and the expression of HIV co-receptors as well as HIV infection were analyzed. The infectibility of DCs with R5 or X4 virus was altered in terms of a reduced integration of viral genome. Both R5 and X4 viruses infected both conditioned and non-conditioned CD4+ T-cells better than DCs, although conditioning did not alter CD4+ T-cell susceptibility to HIV-1 infection.

Several studies have shown that infected seminal leucocytes can cross intact vaginal epithelia to reach the submucosa and the draining inguinal lymph nodes. In this way, cell-associated virus may still establish infection by escaping the high microbicide concentrations in the vaginal lumen. However, virus budding from these migrating leucocytes could be attenuated by prior vaginal drug exposure. Katrijn Grupping and Philippe Selhorst (ITM, Antwerp, Belgium) reported that, in contrast to other antiretrovirals, pre-treatment of HIV-infected PBMCs with M48U1 CD4-mimetic results in a dramatic and prolonged reduction of virion infectivity. This 'memory effect' is related to gp120 shedding from membrane-embedded HIV envelope proteins. Inclusion of M48U1 in topical microbicides may therefore help to reduce the risk of systemic infection by infected cells.

Another EUROPRISE-supported student, Kelly da Costa from St George's Hospital in London, presented research on *in vitro* infection of virus-naïve macaque colorectal explants. She compared SIV superinfection of explants

taken from SIV-infected macaques with either disease progressor or non-progressor phenotype. Colorectal tissues from progressors did not show extra virus production after superinfection challenge, whereas the pattern after challenge of tissues from LTNPs was more variable. While both IgG and IgA total immunoglobulins were secreted from the explanted cultures of SIV-infected macaques, only IgG gp130-specific antibody was detected.

Elisa Saba from the San Raffaele Scientific Institute presented data concerning factors affecting HIV-1 permissibility in a human cervical explant model. The most prominent CD4+ T-cell type in the mucosa was of the effector memory phenotype, and 50% and 80% of the cells expressed CCR5 and CXCR4, respectively. R5 virus efficiently infected the explants over time, while X4 only infected 4/27 donors, despite the high levels of CD4 and CXCR4 in the tissue. Saba also showed that the most permissive donors had high progesterone blood levels, which is in agreement with the literature, suggesting that women on contraceptive pills containing progesterone may be more easily infected than menopausal women. These data support the use of tissue explant models to further characterize factors of importance for the sexual transmission of HIV.

Mucosal vaccination

Given that most HIV-1 transmissions occur during sexual exposure of the genito/rectal mucosa, an ideal HIV-1 vaccine candidate should induce protective antibody responses at the mucosal surfaces [24]. Although some studies have shown that systemic delivery of HIV-1 vaccines can induce HIV-specific immune responses at the mucosa [25,26], findings in mouse models indicate that systemic delivery of HIV-1 vaccines can compromise the quality or avidity of the HIV-specific immune responses at mucosal sites [27].

Paul McKay (Imperial College in London) and coworkers from Queens University (Belfast) have developed a novel antigen delivery device to induce antigen-specific immune responses directly at the vaginal mucosa. A silicone elastomer ring carrier was used to deliver recombinant gp140 antigen adjuvanted with a resiquimod (R848), a TLR 7/8 agonist, into the vaginal cavity of sheep. Antibody responses in sheep receiving only the ring devices were compared to antibody responses in sheep primed intramuscularly with gp140 and R848 followed by insertion of the antigen loaded rings. Results showed that both systemic and vaginal priming induced antigen-specific IgG and IgA responses in serum and vaginal fluid, with the levels of IgA being 30-fold higher at the mucosal surfaces than in serum. It was observed that intramuscular injection did not prime for the development of a mucosal IgA response. Taken together, these data favour the use of this novel ring device for the induction of antigen-specific systemic and mucosal humoral immune responses.

Linda Klavinskis (Kings College, London) described an alternative route of immunization designed to induce mucosal cellular responses. Air-dried dissolvable microneedle arrays were developed to specifically target dendritic cells in the skin, thereby enhancing antigen presentation. Microneedle delivery of a rAd5 vector encoding HIV-1 Gag induced antigen-specific CD4+ and CD8+ T-cell responses in mice, and these responses were as efficient as those induced by subcutaneous or intradermal vaccination. Importantly, the use of this micro-needle array device on the skin allowed the induction of Gag-specific CTL responses in the female genital mucosa. Experiments using transgenic mice with dendritic cells expressing langerin-DTReGFP or CD11c-DTReGFP, demonstrated that conventional $CD11c^{high}$ dermal DCs, but not epidermal Langerhans cells are required for the induction of CD8+ T-cell responses by micro-needle array delivery.

The impact of intranasal immunization on the distribution of antigen-loaded antigen-presenting cells (APCs) and of primed antigen-specific T-cells was studied by Medaglini et al. (University of Siena). Nasal immunization elicited primed T-cells not only in draining lymph nodes but also in non-draining lymphoid sites. They showed, through the use of Fingolimod, a drug that causes lymphocyte sequestration within lymph nodes, that the presence of primed T-cells at distal lymph nodes was due to migration of locally primed T-cells. In contrast to primed T-cells, antigen-loaded APCs could only be detected in the draining mediastinal lymph nodes, and no antigen-loaded APCs were observed in distal lymph nodes. T-cell entry into iliac lymph nodes was found to be CD62L dependent, while CD62L and α4β7 were responsible for homing to the mesenteric lymph nodes. Furthermore, they showed that nasal boosting induces IL-2 producing T-cells, antigen-specific IgG and IgA production and antigen-specific B memory lymphocytes. To further characterise T-cell proliferation, mathematical models were used and it was observed that following nasal immunization, the probability of T-cells entering division is higher and the time of the first division is faster compared to vaginal immunization. Together, these results provide additional information on mucosal vaccination strategies.

The priming of T-cells is a critical event that influences the type and magnitude of immune responses. Further data from Medaglini's group, presented by Fabio Fiorino, evaluated primary CD4+ and CD8+ T-cell activation induced by different mucosal routes of immunization. Mice vaginally and nasally immunized with OVA + CpG were studied for clonal expansion, activation and migration markers at different time points. Both vaginal and

nasal routes of immunization induced high T-cell clonal expansion in the respective draining lymph nodes by day three after vaccination, whilst at day 5, primed T-cells were also detected in distal lymph nodes and in the spleen, with higher percentages detected following the nasal route immunization. The proliferating T-cells were activated and acquired migration properties as indicated by the modulation of CD44, CD45RB and CD62L.

Neutralizing antibodies

HIV-2 is a somewhat understudied virus with a lower pathogenicity and transmission rate than HIV-1. Gulsen Özkaya Sahin and Marianne Jansson from Lund University in Sweden demonstrated that the breadth and potency of neutralizing activity in plasma differ between HIV-1 and HIV-2 infected individuals. Plasma from HIV-2 patients was shown to neutralize HIV-2 isolates at a high frequency, whereas plasma from HIV-1 patients had much lower intratype neutralizing activity against HIV-1 isolates. The intratype neutralizing activity correlated with viral load in patients infected with HIV-1 but not with HIV-2. Hence the neutralizing activity in HIV-2 infection does not seem to require high levels of antigen stimulation, whereas this is more important in HIV-1 infection. Jansson concluded that the viral structure of HIV-2 may promote a more potent humoral immune response and/or HIV-2 itself may be more neutralization sensitive than HIV-1.

Stefania Dispinseri (San Raffaele Scientific Institute) presented data from paediatric infections, correlating disease progression with neutralizing responses. HIV-1 infected children with slow disease progression developed an increasing breadth of neutralizing response over time, whereas fast-progressing children did not.

A similar study, conducted by Katharina Raue, a student from the German Primate Center, analysed the immune responses of infected rhesus monkeys, grouped into controllers or progressors. Animals controlling the infection demonstrated higher immune response than progressors, both humoral and cellular, and the response was directed against Gag proteins and not Envelope.

Not all patients develop broadly neutralizing antibodies; in fact, only 10-30% of HIV-1 infected patients possess antibodies with a strong cross-reactive neutralizing activity and less than 1% have broadly neutralizing antibodies. Zelda Euler from the University of Amsterdam performed a genome-wide association study, to learn more about which genetic markers are involved in the development of antibodies with broadly neutralizing activity. The study included 335 HIV-infected men who have sex with men from the Amsterdam Cohort with known neutralizing activity of their sera. Single nucleotide polymorphisms (SNP), in the major histocompatibility complex gene region, close to MICA and HCP5 genes on chromosome 6, were found to associate with broadly neutralizing activity. HCP5 encodes HLA complex protein 5, and has previously been associated with viral load and disease progression in HIV infected individuals [28,29]. None of the identified SNPs which were associated with broadly neutralizing activity were associated with the clinical course of disease progression. In addition, the association between SNPs or broadly neutralizing activity of antibodies was not related to viral load or CD4+ T-cell count at viral load set-point. Although this finding is remarkable, its application to our understanding of the factors modulating the mounting of an effective immune response is not yet clear.

Marie Borggren, from the University of Lund, compared how CCR5 and CxCR4-using HIV-1 evolve during end-stage disease and how the immune responses correlate with the evolution. The Env proteins of end-stage viruses were often more positively charged and had fewer glycans than virus from the chronic stage of disease. End-stage R5 viruses were shown to use the *trans*-infection route from DC to T-cells less efficiently, which might be due to loss of a specific glycan in gp120.

David Reinhart (Polymun and University of Natural Resources and Life Sciences, Vienna) discussed the switching of the isotypes of anti-HIV-1 IgG antibodies 3D6 and 4B3 recognizing the ectodomain of gp41 to an IgA isotype and their expression in CHO cells in serum-free medium as dimeric IgA. The purification scheme mainly relies on IgA affinity chromatography, using VHH ligands. Unlike previously published protocols, the purification schedule can be used for any IgA irrespective of alpha heavy chains, light chains and their subtypes [30]. These new reagents will allow the study of the influence of antibody isotype on their function with respect to antiviral activity against HIV. Studies are pending to investigate the potential role of IgA during viral exposure at the mucosa.

Enas Sheik-Khalil (University of Lund) described the development of an image-based high-throughput as well as high-content HIV neutralization assay. The assay is based on a plaque reduction assay along with a specialized image analysis tool tailored to analyse neutralization. An additional parameter, mean plaque area, enables the detection of virus fusogenicity to characterize the duration in vitro of neutralization. A decreased plaque size may indicate avidity of neutralizing antibodies. The assay demonstrated equal or higher sensitivity compared to conventional neutralization assays.

Eliciting antibodies

Eliciting new type of antibodies and defining novel epitopes will be critical for future vaccine development. Mark Hassell et al. (NIBSC) have produced novel C clade specific antibodies by priming mice with plasmid DNA encoding either CN54 or ZM96 gp140 genes, followed by a CN54 trimeric gp140 boost, all of subtype C. Applying

the trimeric immunogens, 18 HIV-specific antibodies were successfully generated. However, no novel epitopes were defined. Although all isolated antibodies bound CN54, none were able to recognise and neutralise epitopes that are specific for the trimeric envelope structure.

Another approach to elicit novel antibodies, presented by Pierpaolo Racchiolli et al. (University of Verona), established new anti-HIV-1 antibodies by immunizing mice with CHO cells expressing gp120/gp41 and CHO cells expressing CD4 and CCR5 to generate fusion intermediate structures of the HIV envelope. One hybridoma clone, 10 F12, which showed the highest HIV-1 neutralization activity and lowest cell reactivity, was further investigated and stably produced in CHO cells as a chimeric antibody. Hybridoma and chimeric antibodies both neutralized several HIV viruses.

Other roles for antibodies

HIV infection can be inhibited by both neutralizing and non-neutralizing antibodies via the Fc receptor. Infection of myeloid dendritic cells, mDC, has been shown to be inhibited using both types of antibody. In contrast, Alexandre Lederle, a student from University of Strasbourg, demonstrated that infection of plasmacytoid dendritic cells, pDC, can be inhibited only by neutralizing antibodies, not by non-neutralizing antibodies, despite the expression of Fc receptors on the surface of pDC.

As discussed previously, transmission of HIV-1 via mucosal tissues during sexual intercourse globally represents the main route of infection. Immature dendritic cells reside in the genital mucosal tissues and are believed to be among the first cells to be in contact with HIV. An infected DC may act as a "Trojan horse" for HIV and further transmit viruses to primary CD4+ T lymphocytes. Antibodies that inhibit such viral transfer could prevent HIV infection. Bin Su from the University of Strasbourg addressed this hypothesis and demonstrated that neutralizing antibodies could efficiently inhibit the transfer of several R5 HIV-1 strains from primary DCs to autologous CD4+ T lymphocytes. Furthermore, he showed that neutralizing as well as non-neutralizing antibodies could decrease HIV-1 replication in DCs. Induction of both types of antibodies directly at mucosal sites may thus be beneficial when designing a future vaccine.

Besides neutralization, HIV-specific antibodies could thus have an impact on protection against HIV-1 transmission in several ways. Such HIV-specific antibodies could target not only free virions, but also infected cells, as well as the HIV transfer from infected cells to other cells. In order to understand the contribution of antibodies induced against these targets and especially the role of Fc-mediated protection, Marina Biedma from the University of Strasbourg investigated several inhibitory functions of anti-HIV IgGs. It was found that non-neutralizing inhibitory antibodies (NNIAbs) could mediate inhibitory activities such as ADCC or phagocytosis. Indeed, it was demonstrated that NNIAbs such as 4B3 and 246D were highly efficient in capturing free virions when compared to neutralizing antibody 2G12, 4E10 or 2F5. Furthermore, NNIAbs 4B3 and 246D revealed Fc-mediated inhibitory activities on viral replication in macrophages and DCs similar to some neutralizing antibody. When applied locally at the vaginal site of macaques, these NNIAbs were able to significantly decrease the peak viral load following vaginal experimental challenge with SHIV SF162P3. These results suggest that NNIAbs may be important in controlling viral infections.

In addition to broadly neutralizing antibodies, other targets for antibodies with alternative antiviral function are being explored. Donato Zipeto from the University of Verona described the identification of one such new target by unravelling the association between HLA-C and gp120. Using bimolecular fluorescence complementation analysis, it was shown that HLA-C competes for binding between β_2m and gp120. Furthermore, association of gp120 with HLA-C happens early in the endoplasmic reticulum and continues during passage through the Golgi apparatus and early endosomes, until presentation at the cell membrane. It was found that HLA-C specifically incorporates into HIV-1 envelopes and increases viral infectivity by increasing viral envelope fusion with R5 and X4 HIV strains. A compound or antibody that could disturb this association might decrease viral infectivity. Furthermore, antibody to HLA-C displayed on the virusor virus-infected cell surface might participate in ADCC.

Innate immunity against HIV

In a setting where ongoing attempts to develop an HIV-1 vaccine face significant challenges, investigation of innate immune factors and the host response to infection are crucial for the development of novel prophylactic strategies against HIV/AIDS. Innate responses not only influence mucosal transmission and establishment of initial infection foci, but may also have an impact on the subsequent level of ongoing viral replication and rate of disease progression [31]. Studies were presented to explain the contributions made by components of the host innate response to HIV acquisition and spread versus its control.

Magarita Bofill (IrsiCaixa, Barcelona) has previously described that co-stimulation of mononcytes with IL-12 and IL-18 triggers the survival and differentiation of monocytes to macrophages, important players in innate immunity [32]. As an advance on this work she demonstrated that the treatment of differentiated macrophages but not monocytes with IL-12 and IL-18 induces production of IFN-γ in an antigen-independent manner. Absence of proviral or integrated DNA in target macrophages identified the inhibitory effects of IL-12 and IL-

18 as entry events. Furthermore, IL-12 and IL-18 induced Apobec3G up-regulation in these target cells.

Interferon (IFN)-induced intracellular antiviral proteins, referred to as restriction factors, are capable of interfering with retroviral replication at various steps of the viral life-cycle. Elisa Vicenzi's group (San Raffaele Scientific Institute) has exploited cell clones isolated from the U937 promonocytic cell line, either permissive or non-permissive for HIV-1 replication. Data revealed that among known HIV-1 restriction factors, the IFN-inducible Tripartite motif-containing protein 22 (TRIM22) was the only factor constitutively expressed in non-permissive but absent in permissive U937 cells. Conversely, stable knock-down of TRIM22 rescued the non-permissive cell line, while over-expression of TRIM22 in a permissive cell line inhibited HIV-1 replication. A similar inhibitory effect of TRIM22 was confirmed in human A301 T-cells. Most likely, nuclear TRIM22 impairs HIV-LTR driven transcription in a Tat- and NF-κB-independent manner [33].

Adjuvants

Specific adjuvant formulations triggering innate immune signalling pathways can improve the potency and quality of adaptive immune responses. Therefore, many studies are focusing on the development of novel adjuvants that might increase the immunogenicity of HIV-1 antigens.

Annette Sköld (Karolinska Institutet) presented a poster on the use of single-stranded DNA oligonucleotides (ssDNA ODNs) as an inhibitor of detrimental TLR3 responses. Results showed that the addition of ssDNA ODNs inhibited the TLR3-triggered cytokine release and maturation of human DCs. In addition, ssDNA ODNs blocked TLR3-mediated release of pro-inflammatory cytokines in the airways of cynomolgus macaques. In summary, while the use of ssDNA ODN might open novel perspectives for clinical treatment of TLR3-mediated inflammatory disorders, it was clear that combining ssDNA ODN with TLR3 agonists may be detrimental for the efficient induction of immune responses.

In work presented by Quentin Sattentau (University of Oxford), the nucleic acid transfection reagent polyethyleneimine (PEI) was shown to exhibit potent mucosal adjuvant properties. Intranasal administration of the influenza HA antigen, adjuvanted with PEI, protected mice against viral infection and disease. Moreover, in the induction of protective responses, PEI was superior to other known mucosal adjuvants, including CpG. Similar results were obtained for herpes simplex virus 2 (HSV-2) gD and HIV-1 gp140 antigens. It was shown that PEI forms complexes with antigens, thereby targeting DCs. Indeed, such particulate antigen systems are similar in size to microorganisms, and are consequently efficiently taken up by phagocytic cells, including DCs. In addition, PEI was shown to activate innate immune responses, including inflammasome- and MyD88-dependent pathways.

HIV/SIV pathogenesis and disease progression

Correlates of pathogenicity

Based on previous observations that a high frequency of adenosine nucleotide in HIV/SIV RNA correlates with the pathogenicity of a lentivirus, a study presented by Frederic Tangy (Institut Pasteur) focused on the effect of the viral A/C/G/T nucleotide ratio on stimulation of innate immune responses. Synthetic SIVs genomes were designed without modifying regulatory elements or sequences of amino acids. The first SIV was subjected to codon changes of *gag* and *pol* genomic regions, which resulted in a dramatic decrease in replicative capacity. The second SIV harboured only a *pol* optimized gene, which resulted in a reduced ability to stimulate type-I interferon *in vitro* in cellular assays. Nucleotide patterns (without changed amino acid patterns) may thus play a role in the induction of innate immunity and in virulence.

A EUROPRISE student Aneela Javed (German Primate Center) investigated factors correlated with CD8+ T-cell Non-Cytolytic Antiviral Activity (NCAA) in SIV-infected rhesus macaques. NCAA is associated with reduced transmission of HIV and slow disease progression. NCAA + and NCAA- animals were identified using an in vitro viral inhibition test. Differentially expressed genes in CD8+ T-cells from NCAA + and NCAA- animals were investigated using microarrays. FAM26F, translating a membrane-bound protein with undefined function, was identified as a candidate gene. Furthermore, the expression of FAM26F is negatively correlated with viral load, while positively correlated with the expression of two cytokines, IP-10 and MX. Further studies in humans are ongoing.

T-cells

Not all individuals have the same T-cell profile, and co-infection with HIV and other viruses, bacteria or parasites may influence the target T-cell pool for HIV. Understanding the co-infection responses and the consequence for the HIV infection could affect individualized treatment. William Paxton from the University of Amsterdam compared the *mycobacterium tuberculosis* (MTB)- and cytomegalovirus (CMV)-specific CD4 T-cell responses in patients co-infected with HIV. His results demonstrate that the CD4 T-cell population is skewed in these patients, with a persistent CMV-specific T-cell response but a MTB-specific response that was rapidly depleted. The CMV-specific CD4 T-cells had a mature phenotype, with high MIP-1β and low IL-2 producing cells. In contrast, the MTB-specific CD4 T-cells were less mature, with more IL-2 production and less MIP-1β. These findings confirm that HIV preferentially infects IL-2 producing T-cells, and that MIP-1β producing cells are more resistant.

B-cells

Nicolas Ruffin from the Karolinska Institutet investigated the effect of HIV infection on the B-cell compartment of HIV-infected patients. He found that the levels of sCD14, a marker of microbial translocation, correlated with B-cell activation and loss of resting memory B-cells. sCD14 was associated with higher IL-21R expression on resting memory B-cells and the B-cells bearing IL-21R were shown to be more susceptible to apoptosis. This finding provides an important insight into how the humoral arm of the immune system may be affected by the microbial translocation that occurs during HIV-1 infection.

Simone Pensieroso (San Raffaele Scientific Institute) examined the hypothesis that highly efficient long-term antiretroviral therapy could quantitatively restore all B-cell subsets. In particular he examined resting memory B-cells, which are responsible for secondary immune responses and are severely depleted in HIV-infection. After failure of previous therapies, 30 HIV-1 infected multi-drug experienced patients were treated with Highly Active Antiretroviral Therapy (HAART including Raltegravir) and followed for 144 weeks. 72 age-matched uninfected individuals were used as healthy controls. An effective Raltegravir-including regimen was shown to restore all B-cell subpopulations with the exception of the resting memory B-cell subset when monitored for up to three years. A longer follow-up study might determine whether resting memory B-cells need even more time to recover.

New methods and reagents

Measurement of immune responses

One of the objectives of the EUROPRISE consortium has always been to develop new techniques, assays and standard reagents and make them available to researchers around the world to help accelerate research on HIV vaccines and microbicides.

A new assay developed by Mabtech with support from EUROPRISE is the FluoroSpot assay, which is based on the same principle as the well-known ELISpot assay. The FluoroSpot assay is characterized by high sensitivity and specificity and can be used to analyse simultaneous secretion of cytokines by T-cells. It also allows the quantification of singly and dually secreting cells. Clearly, this can provide important information about the quality of a specific T-cell response. So far, combinations of IFN-γ /IL-2, IFN-γ /IL-5, IFN-γ /IL-13, and IFN-γ /IL-17A have been developed. Furthermore, it is possible to detect IgG and IgA secreted by B-cells in a single well and analysis can be performed on mice, monkey and human cells.

JPT Peptide Technology, a company in Germany, presented their technology platform for peptide microarrays. They have generated a versatile peptide library spanning the immunogenic fractions of the HIV proteome with high coverage across viral clades. JPT have been successful in generating overlapping peptide sets representing all immunogenic regions of target HIV proteins. The final library of 5572 different 15-mer peptides achieves coverage of 86% of all 3578 M-group and recombinant sequences of p24 as well as coverage of 50% of the published 2248 sequences of gp160. A major advantage of this library design is the ability to condense and cover the immense complexity of the immunogenic domains of the HIV proteome with only 5572 overlapping peptides that can be accommodated on a single high-density peptide microarray for clinical HIV research. These peptide microarrays have several applications, such as biomarker identification, vaccine target identification, immuno-monitoring, mapping B-cell responses, and profiling of enzymatic activity. Monitoring the antibody response in detail before and after vaccination in patient samples is crucial. In this context, the peptide microarray, which gives insights into the antigen-antibody interaction on the sub-protein level, may complement other assays which rely on the entire antigen. Nevertheless, the technology is restricted to the extent that it can map only linear and not conformational epitopes.

Reagents

The Division of Retrovirology at the NIBSC in the UK produces a variety of biological standards and reference materials designed as quality controls for HIV diagnostic (serological) assays and nucleic acid-based amplification techniques. The HIV-1 and HIV-2 Internal standards (IS) developed by NIBSC have been used to standardize both commercial and in-house assays. NIBSC has also developed a first genotype panel containing genotypes A-H, as well as HIV-1 working reagents calibrated against internal standards to support serology-based diagnostic assays. A selection of recently transmitted subtypes and subgroups of HIV-1 strains has been assembled to create a panel with a great variety of viral stocks. The viral isolates were collected from HIV-1 infected patients and expanded in PHA stimulated PBMCs, whereafter they were characterized using RT-PCR and phylogeny. The stocks are held by NIBSC and are available to all HIV researchers.

The Center For AIDS Reagents (CFAR) at NIBSC has been able to support HIV/AIDS research through the supply of reagents to scientists in Europe and worldwide. With the help of 173 novel materials provided by the EUROPRISE collaborators, CFAR now has a repository of over 6000 reagents. They have also successfully produced novel, lyophilized cell preparations for detecting and quantifying CMI assays. The availability of such useful materials is a legacy of success for the EUROPRISE group.

Concluding remarks

The fifth EUROPRISE Network annual conference was held in Prague in November 2011 in an atmosphere of

optimism. Imaginative and novel strategies to be used in HIV prophylaxis and intervention were discussed.

The scientists involved in this Network of Excellence have participated in over 200 multi-author papers, weekly bulletins have been provided within and outside the network, and HIV/AIDS reagents have been provided to colleagues worldwide. The Network´s extremely successful students have bound the research groups together, formed networks of their own and brought an immeasurable feeling of collaboration, sharing of ideas and positive aspects of future research collaboration to the Network. Such integrated science will permit continued developmental research from discovery to clinical trials, some of which are described in this review.

The co-usage of vaccines and microbicides is unique; the Network has brought forward research in this area and is now also starting preclinical and clinical trials with the aim of combining the two. The meeting in Prague was focused on collaborative work between partners, in particular scientific work performed and presented by the Network's PhD students. The abilities of students to perform and present up-to-date science promise a bright future for the next generation of HIV researchers in Europe.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
NR, MB, ZE, FF, KG, DH, AJ, KM, CP, DR, ES, ESK, AS and SZ were in charge of the writing of dedicated chapters covering the various sessions of the conference. RS, GS, FG and BW organized the sessions and the writing, and corrected and revised the manuscript. All authors read and approved the final manuscript.

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