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ANTIBODY EVOLUTION AND REPERTOIRE DEVELOPMENT

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ABBREVIATIONS

aa	amino acid
AD	antigenic domain
AID	activation-induced cytidine deaminase
C	constant
CDR	complementarity determining region
D	diversity
DNA	deoxyribonucleic acid
Fab	fragment, antigen binding
Fc	fragment, crystallizable
FR	framework region
Fv	fragment, variable
gB	glycoprotein B
H	heavy
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
Ig	immunoglobulin
IGH[1]	immunoglublin heavy
IGK	immunoglublin kappa
IGL	immunoglublin lambda
IMGT	the international immunogenetics database
J	joining
L	light
mRNA	messenger RNA
RAG	recombination activating gene
RNA	ribonucleic acid
RSS	recombination signal sequence
scFv	single-chain antibody fragment variable
V	variable

[1] Gene names and abbreviations used in this thesis are according to the official IMGT/HUGO nomenclature (IMGT, the international ImMunoGeneTics database, LEFRANC & LEFRANC, 2001).

1 INTRODUCTION

Antibodies, also known as immunoglobulins, are key players of the immune system in higher vertebrates, which provide a defense against potentially lethal threats from the environment. They were originally discovered in the 1890s, when von Behring and Kitasato found substances in sera they termed antitoxins for their capacity to neutralize or destroy bacterial toxins. The more general term antibodies was soon adopted, and in 1901 von Behring was awarded the first Nobel Prize in Medicine for his discovery (SILVERSTEIN, 1999).

The origin, structure, and diversity of antibodies and the multitude of processes they are involved in, both as cellular receptors and as secreted proteins, have since the days of von Behring and Kitasato been thoroughly studied. Today we know that antibodies are produced by a particular set of lymphocytes called B cells, and that their capability of recognizing an essentially infinite number of antigens is the result of recombination of a limited set of gene segments followed by somatic diversification processes (TONEGAWA, 1983). The complete repertoires of germline genes that encode antibodies in both humans as well as other vertebrates have recently also been elucidated and are available on the worldwide web (IMGT, the international ImMunoGeneTics database, http://imgt.cines.fr, LEFRANC & LEFRANC, 2001), thereby providing invaluable information to both immunologists and molecular engineers. In addition, the structure of practically every part of the antibody molecules has been delineated, and today we are on verge of being able to design an antibody of defined specificity from scratch (MOREA et al., 2000).

Besides their importance in the natural immune defense, the potential of antibodies as reagents in biological chemistry and diagnostics, and as therapeutic agents against infectious diseases as well as cancer is more and more being appreciated (HUDSON, 1998; BORREBAECK, 2000; BORREBAECK & CARLSSON, 2001). The use of antibodies for therapy has been aided by the discovery of antibody technologies, such as the hybridoma technology (KÖHLER & MILSTEIN, 1975), which allowed for the production of large quantities of monoclonal antibodies with defined specificities. The original use of murine antibodies was severely hampered by their inability to evoke human effector

functions, and, more important, by their immunogenicity in humans. The development of phage display (SMITH, 1985), with its subsequent use for antibody phage display (reviewed in WINTER et al., 1994; HOOGENBOOM et al., 1998), has completely changed this. Together with recombinant antibody technologies (DALL'ACQUA & CARTER, 1998; HUDSON, 1998) phage display and related techniques have made it possible to generate fully human antibodies of a multitude of formats against virtually any antigen, including human self antigens. Furthermore, antibody phage display has also provided the tools for studying antibody repertoire development in a number of important malignancies, such as auto-immune diseases, allergies, and infectious diseases, which may facilitate the development of possible therapeutic modalities and vaccines.

Despite the tremendous advances in all fields regarding antibodies, the understanding of the processes that shape antibody responses *in vivo* is far from complete, as demonstrated by the constant discovery of novel factors and mechanisms that may influence the outcome of these processes. In this thesis, which is based on five original papers, I present work that deals with aspects of the evolution of antibodies (Papers I and II) and the development of antibody repertoires (Papers III-V). Paper I describes the identification of patterns in the inherited repertoire of human genes encoding antibodies that target them with insertions and deletions during the maturation of an antibody response. Paper II is a follow-up of this finding and deals with the functional consequences of such insertions and deletions, and the possibility to use them in antibody engineering to create antibodies against specific targets. The remaining papers describe how a repertoire of antibodies develops against a weakly immunogenic epitope on a human virus, and what the parameters are that determine the neutralizing capacity of such antibodies. The human cytomegalovirus, which has been a long-standing interest at the Department of Immunotechnology, was chosen as a model system for these studies because antibodies reactive with this virus have a biological significance and a possible use in therapy. The results may provide insights into the development of similar antibody repertoires in general, and are also discussed in the context of vaccine development.

2 ANTIBODY STRUCTURE AND FUNCTION

Antibodies, also called immunoglobulins, are serum glycoproteins proteins that are produced in vertebrates as a response to challenge by foreign matter, so-called antigens. Antibodies have two distinct functions; the recognition of foreign antigens, and the recruitment of effector systems by binding to cellular receptors or other effector molecules. In order to be able to perform both functions, the antibody molecule is organized in a variable region and a constant region. The variable region is, as the name suggests, highly diverse in sequence and structure between different antibodies in order to be able to recognize the multitude of antigens the immune system has to deal with. The constant region is, on the other hand, more conserved between different antibodies as they all have to be able to recruit the same effector systems.

2.1 General Structure

Antibodies are among the best studied of all proteins, and antibody structure has been extensively reviewed over the years (AMZEL & POLJAK, 1979; DAVIES et al., 1990; WILSON & STANFIELD, 1993; PADLAN, 1994; FRAZER & CAPRA, 1999). All antibodies have the same basic composition of four polypeptide chains, two identical heavy chains and two identical light chains, which are linked together by disulphide bridges. The general shape of the molecule is believed to be that of a distorted Y or T, a notion that has also been supported by the first structures of intact murine antibodies (HARRIS et al., 1992; HARRIS et al., 1998). A very recent structure of the first intact human antibody has, however, showed that an antibody may also display extreme asymmetry, suggesting that antibody molecules may be extraordinary flexible (SAPHIRE et al., 2001).

There are five classes of human heavy chains; γ, μ, α, δ, and ε, which determine the functional class or isotype (IgG, IgM, IgA, IgD, and IgE, respectively) of the antibody produced. In contrast, there are only two human light chain classes; κ and λ, which are functionally indistinguishable (BENGTÉN et al., 2000). The IgG and IgA isotypes are further divided into four and two subclasses, respectively, due to the existence of multiple classes of γ and α heavy chains, whereas only one type of chain exists for the other isotypes. Each

isotype determines the mode of elimination of the captured antigen or the location where the immunoglobulin is delivered and accumulated. IgG is by far the most abundant isotype in serum, and due to it having the simplest structure of all isotypes, it is often used to represent the basic immunoglobulin structure (FIG. 1). The light chain classes may combine with any of the heavy chain isotypes, but in any one immunoglobulin molecule, both chains are of the same type. Each immunoglobulin chain folds into separate domains comprising approximately 110 amino acids, which share a common folding pattern, the so-called immunoglobulin fold (POLJAK et al., 1973). This fold is best described as two twisted, anti-parallel β sheets that are packed tightly against each other, thereby enclosing a hydrophobic core. The domains are further stabilized by a disulphide bond between the two sheets. Light chains fold into two such domains, and heavy chains into four or five. The amino-terminal domain of both heavy and light chains is termed variable (V), as different antibodies display extensive sequence variability in this region. The carboxy-terminal domains are more conserved in sequence and are consequently referred to as constant (C).

Variable domains are formed by nine β strands and constant domains by seven strands, and the strands in different sheets are joined by loops of different lengths and conformations. The loops in the V domains are involved in antigen binding (see below), whereas the loops of the C domains have generally not been attributed with any particular functions. However, it has been noted that these loops are actually more diverse in length than the loops in the V domains (PADLAN, 1994; FRAZER & CAPRA, 1999). It may be possible that the loops are responsible for the differences in effector functions that exist between the different isotypes (BENGTÉN et al., 2000). The γ, α, and δ chains fold into three C domains (CH1, CH2 and CH3), with a flexible hinge region between domains CH1 and CH2 (FIG. 1). Heavy chains of the μ and ε classes fold into four C domains (CH1, CH2, CH3, and CH4), where CH2 replaces the hinge region, and CH3 and CH4 correspond to CH2 and CH3, respectively, in the three-C domain isotypes.

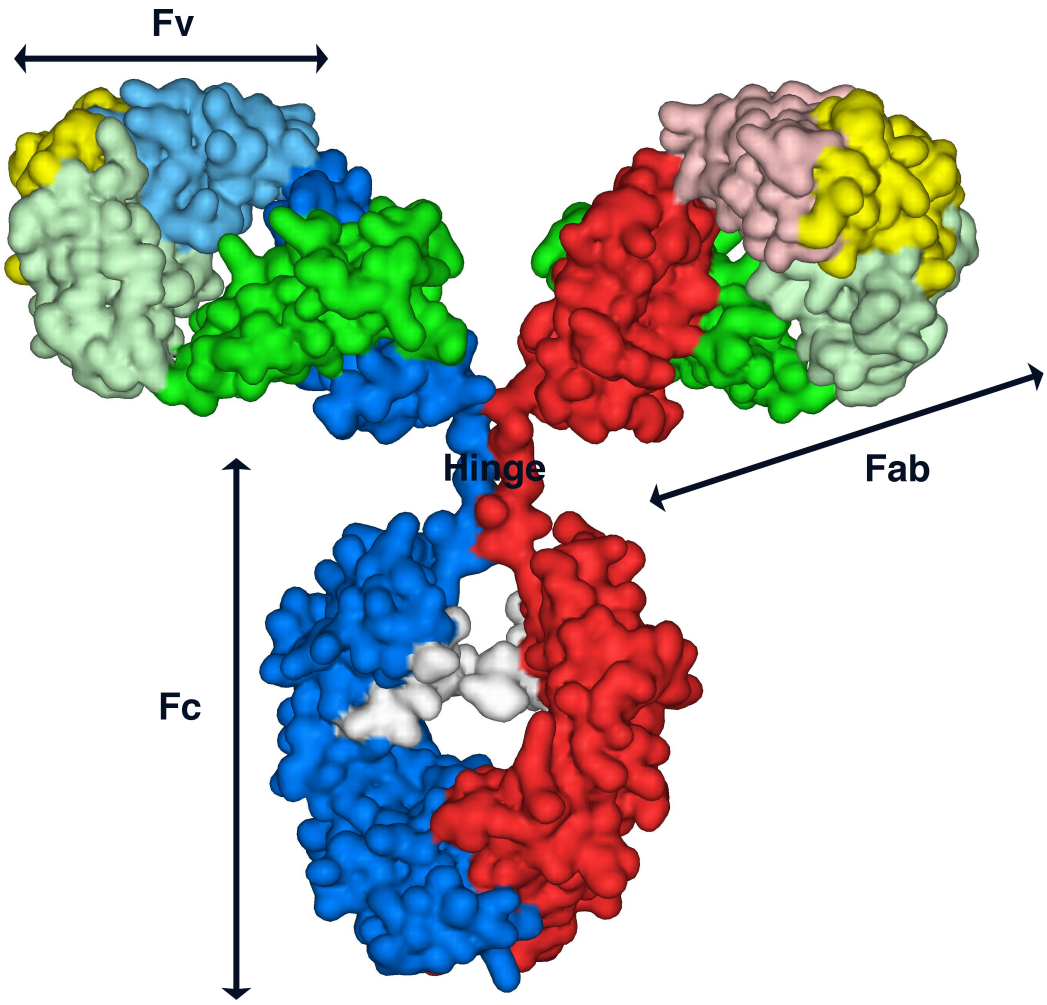

FIG. 1. Structure model of an IgG molecule. The PDB (Protein Data Bank) format file for the antibody structure model was obtained from Mike's Immunoglobulin Structure/Function Home Page (http://www.path.cam.ac.uk/~mrc7/pdb/IgG1.pdb), and the image was generated using ViewerLite 5.0. The two heavy chains are indicated in blue and red, whereas the light chains are coulored green. The variable domains of all chains are indicated in lighter colours, and the surfaces formed by the CDR, i.e. the antigen-binding sites, are yellow. The carbohydrate moieties between the CH2 domains are indicated in white.

The modular three-dimensional structure of immunoglobulins lends itself well to antibody engineering purposes, as each individual domain retains its native structure when isolated from the whole antibody and the function of each domain can be recovered (reviewed in HUSTON et al., 1996). It was originally discovered that treatment of IgG with the proteolytic enzyme papain (which cleaves in the hinge region) would produce two identical fragments that could bind antigen – Fab (Fragment, antigen binding) and a third fragment which cannot bind antigen – Fc (Fragment, crystallizable) (FIG. 1). The Fab fragment consists of one complete light chain linked by a disulfide bridge to a fragment of the heavy chain consisting of VH and CH1. The Fc comprises CH2 and CH3 from both heavy chains similarly linked by disulfide bonds. The Fv comprises VH and VL domains and is the smallest fragment that retains the full monovalent binding of the intact parent immunoglobulin. As Fv are not easily produced by proteolysis, recombinant single-chain Fv (scFv) molecules, where VH and VL are joined by a polypetide linker (HUSTON et al., 1988), are usually used to mimic the Fv.

2.2 The Antigen-Binding Site

The residues of the variable domains can be divided into hypervariable or complementarity determining regions (CDR) and framework regions (FR) based on the observed sequence variation (WU & KABAT, 1970). Each variable domain contains three CDR (CDR1-CDR3), which are flanked by the relatively conserved framework regions (FR1-FR4). The FR form the β-sheet structure of the variable domain immunoglobulin fold from which the CDR protrude and make up the loops at one end of the sheets. The pairing of the VL and VH domains brings together the six CDR loops to form the antigen-binding site (POLJAK et al., 1973; PADLAN, 1994) (FIG. 1).

A number of studies of antibody structures have shown that five of the six CDR loops generally adopt only a limited number of backbone conformations, so-called canonical structures, and that these conformations can be predicted from the antibody sequence (CHOTHIA & LESK, 1987; CHOTHIA et al., 1989; 1992; TRAMONTANO et al., 1990; WU & CYGLER, 1993; TOMLINSON et al., 1995; GUARNÉ et al., 1996; MARTIN & THORNTON, 1996; AL-LAZIKANI et al., 1997).

These conformations are mainly determined by the length of the loops and by the presence of certain key residues, such as heavy chain residue 80,[2] which are involved in the packing of the loops against the framework (TRAMONTANO et al., 1990; MARTIN & THORNTON, 1996; AL-LAZIKANI et al., 1997). The third loop of the VH domain (CDRH3) is much more variable in sequence, length and conformation than the other loops, and it is thus much more difficult to predict its structure. However, as the CDRH3 loop is very often crucial in determining the specificity of an individual antigen-binding site (SEGAL et al., 1974; SHARON, 1990), efforts have been made to predict the structure of these loops as well. The result of these have been the formulation of rules that describe the backbone conformation of the residues proximal to the framework (the "torso" or the "base" of the loop) (SHIRAI et al., 1996; 1999; MOREA et al., 1998; OLIVA et al., 1998). Some generalizations regarding the conformation of the residues at the apex of short CDRH3 loops exist as well (MOREA et al., 1998; SHIRAI et al., 1999).

The canonical structures do not occur in all combinations. For instance, in human heavy chains, three CDR1 structures and four CDR2 structures have been observed, but of the possible 12 combinations, only six are actually found in the germline gene repertoire (CHOTHIA et al., 1992; AL-LAZIKANI et al., 1997). Furthermore, Vargas-Madrazo *et al*. found by analyzing the sequences of antibodies with known specificities that only ten of the approximately 300 possible combinations of CDRL1-L2-L3-H1-H2 canonical structures account for 87% of the sequences (VARGAS-MADRAZO et al., 1995).

The six CDR form a continuous surface (FIG. 1) of approximately 2800 Å^2, the topography of which varies greatly due to the variation in sequence and length of the CDR loops (PADLAN, 1994). It has been known for quite some time that the gross geometry of the antigen-binding site correlates with the type of antigen recognized by the antibody. Three different types of paratopes have been described; cavity (for haptens), groove (for peptides, DNA, and carbohydrates), and planar (for proteins) (WILSON et al., 1991; WILSON & STANFIELD, 1993; WEBSTER et al., 1994; MACCALLUM et al., 1996). These differences in paratope

[2] Antibody sequence numbering and CDR definitions used throughout this thesis are according to the IMGT unique numbering (IMGT, the international ImMunoGeneTics database, LEFRANC & LEFRANC, 2001).

topography are also reflected in the buried surface area of the antigen-binding site upon complex formation with the antigen, which increases in the order cavity < groove < planar. Antibody-hapten complexes have a buried surface area of the antibody of 100-300 $Å^2$, antibody-peptide complexes 460-600 $Å^2$, and antibody-protein complexes 600-900 $Å^2$ (WILSON & STANFIELD, 1993; WEBSTER et al., 1994; BRADEN & POLJAK, 1995; DOKURNO et al., 1998).

The antigen-binding specificity of an antibody is primarily determined by the conformation and relative disposition of the different CDR, and by the nature and orientation of the side chains of the residues in the CDR (PADLAN, 1994). A very thorough analysis of contact residues in antibody-antigen complexes of known structure by MacCallum *et al*., where the involvement in antigen binding of each residue within the CDR was assessed, showed that antigens tend to bind to residues located at the center of the antigen-binding site where the six CDR meet (MACCALLUM et al., 1996). Most antigens are contacted by at least five of the CDR, with heavy chain CDR making somewhat more extensive contacts with the antigen (WILSON & STANFIELD, 1993; BRADEN & POLJAK, 1995; MACCALLUM et al., 1996). CDRL2 only rarely contributes to antigen binding. The minimum number of CDR loops that are involved in binding to even the smallest antigen is four (WILSON & STANFIELD, 1993). However, isolated murine and camelid VH domains have also been shown to be able to bind antigen specifically (WARD et al., 1989; MUYLDERMANS et al., 2001), indicating that three CDR are enough for effective antigen recognition. In fact, crystallographic data have shown that in specific cases, only one CDR is involved in antigen contact (DESMYTER et al., 2001; MUYLDERMANS et al., 2001). Studies with cyclic peptides have also shown that isolated CDRH3 sequences can by themselves mimic the recognition pattern and sometimes also the function of the parent antibody (LEVI et al., 1993; DITZEL et al., 1996; DENG & NOTKINS, 2000). In addition to contributions from the different CDR, conformational changes in both the antigen and the antigen-binding site have also been shown to be involved in the complex formation. In the antibodies, these changes range from simple side-chain movements to rearrangements of individual CDR loops, in particular CDRH3, and VH-VL displacements (WILSON & STANFIELD, 1993; 1994; DAVIES & COHEN, 1996).

The relationship between the canonical loop structures and the overall topography of the antigen-binding site has been further investigated by Vargas-Madrazo *et al*., who have found that the combination of canonical loop structures corresponds to the antigen recognized by the antibody (VARGAS-MADRAZO et al., 1995; LARA-OCHOA et al., 1996). They were able to define two distinct classes of antibodies; those that bind a specific class (size) of antigen, and those that are multi-specific. Among the specific classes, the antibodies that bind small molecules have cleft-like antigen-binding sites formed by long CDRH2 and CDRL1 loops, whereas antibodies that bind large molecules tend to have flatter antigen-binding sites due to short CDRH2 and CDRL1 loops. The CDRH3 loop does not appear to influence the gross topography of the binding site of the specific classes, whereas it is an important determinant of the shape of the binding site in the multi-specific antibody classes (VARGAS-MADRAZO et al., 1995; LARA-OCHOA et al., 1996). An interesting observation in connection with this is that human antibodies specific for protein antigens tend to have longer CDRH3 loops than those found in random repertoires of antibodies (OHLIN & BORREBAECK, 1996). It is possible that this is a consequence of selection for longer CDRH3 loops that would provide more contacts with the large protein antigens and thus also higher affinity of the interaction.

3 GENERATION OF ANTIBODY DIVERSITY

As mentioned briefly in the introduction, antibodies are encoded by different sets of germline gene segments. The heavy chain V domains are encoded by three gene segments (V, D (diversity), and J (joining), respectively), and the light chain V domains by two (V and J). These gene segments are separated by thousands of basepairs in the genome, but are brought together by a recombination machinery and joined into functional heavy and light chain genes during the differentiation from a hematopoietic stem cell to a mature B cell (TONEGAWA, 1983). The CDR1 and CDR2 of both the heavy and the light chains are encoded by the V genes, whereas CDR3 is encoded by the assembled V and J segments. In the case of the heavy chain, a D segment is also incorporated between the V and J segments and encodes the central part of CDR3.

Following productive V gene rearrangement, the functional immunoglobulin genes in the B cells of humans and mice are subjected to further types of genetic modification upon antigen encounter. The first, class switching, is a region-specific but largely non-homologous recombination process, which leads to a change in constant region of the expressed antibody. The second one is somatic hypermutation, which greatly contributes to diversifying the information encoded by the germline gene segments. Recently, it has been shown that processes such as secondary rearrangements and gene conversion may also contribute to this diversification.

3.1 V(D)J Recombination

The first step in the creation of antibody diversity is the recombination of gene segments that encode the V domains, which is commonly referred to as V(D)J recombination. The process of recombination occurs at specific recombination signal sequences (RSS), which consist of a heptameric sequence and a nonameric sequence that are separated by a spacer of 12 or 23 base pairs. Functional recombinations occur only between gene segments carrying spacers of different lengths, one carrying a 12 basepair spacer and the other a 23 basepair spacer (referred to as the 12/23 rule) (TONEGAWA, 1983). Besides the diversity that is created by the combinatorial joining of different gene segments,

additional diversity is produced by the imprecise recombination of these segments and by the introduction of junctional diversity.

A mechanism involving DNA double strand breaks and the formation of closed hairpin loops at the ends of the V, D, and J segments has been proposed to be responsible for the recombination (BASSING et al., 2002). The products of the recombination activating genes-1 and –2 (*RAG-1* and *RAG-2*) have been shown to be an absolute requisite for the initiation of V(D)J recombination, although their exact function is still unknown. The RAG proteins produce single strand breaks between the coding regions and the flanking RSS, which leads to the formation of hairpin loops in the V(D)J ends and blunt RSS ends. The opening of these hairpin loops and the subsequent processing of the ends before joining leads to the addition or deletion of nucleotides at the junctions. The deletions are suggested to be caused by an exonuclease that nibbles the ends after the opening of the loops. The added nucleotides are either untemplated nucleotides caused by the action of terminal deoxynucleotidyl transferase (so-called N region nucleotides), or palindromic sequence nucleotides (P region nucleotides) caused by asymmetric opening of the hairpin loops and the absence of exonuclease activity (BASSING et al., 2002).

3.2 Class Switching

Mature B cells, which have completed functional V(D)J recombination, express B cell receptors on their surfaces and migrate to secondary lymphatic organs, such as the spleen and lymph nodes, where they encounter antigens. The activation of B cells by antigen stimulation causes them to proliferate and form germinal centers where the next steps in the generation of antibody diversity take place; isotype switching, somatic hypermutation, and, in some cases, secondary rearrangements.

The B cells that enter the secondary lymph organs express antibodies of the IgM and IgD isotypes. Isotype class switching is the process of replacing the original constant region gene of the heavy chain with that of one of the IGHA, IGHG, or IGHE genes, leading to an isotype switch to either IgA, IgG, or IgE, respectively, without changing the specificity of the resulting antibody (HARRIMAN et al., 1993).

3.3 Somatic Hypermutation

Somatic hypermutation of antibody V genes was first described more than thirty years ago (WEIGERT et al., 1970) and serves as the basis for affinity maturation during immune responses. During this process, mutations are accumulated in the V genes of antibodies, and B cells that express high-affinity antibodies are selected by competition for limited amounts of antigen carried on follicular dendritic cells (BEREK et al., 1991; MACLENNAN, 1994; RAJEWSKY, 1996). The exact mechanism of somatic hypermutation is still unknown, but evidence suggests the involvement of an error-prone DNA repair system (JACOBS & BROSS, 2001). What is known is that the process is linked to transcription (PETERS & STORB, 1996), and that it probably involves a DNA double strand break (BROSS et al., 2000). Furthermore, the process targets bases non-randomly. It has been demonstrated that mutations are accumulated in the CDR, while the FR are relatively conserved (BETZ et al., 1993). Primary sequence motifs, such as the RGYW motif or its complement, WRCY, have been identified as hotspots for the hypermutation process (ROGOZIN & KOLCHANOV, 1992; DUNN-WALTERS et al., 1998; DÖRNER et al., 1998b). Consistent with these findings, the CDR have also been shown to display a bias for AGY serine codons over TCN codons, which are preferred in the FR, resulting in an increased targeting of mutations to serines in the CDR (WAGNER et al., 1995). Furthermore, it has been shown that the codons used in CDR are more susceptible to replacement mutations than codons used in FR (CHANG & CASALI, 1994), which provides an additional explanation to the accumulation of mutations in the CDR.

Recently, Muramatsu et al. discovered a new cytidine deaminase (Activation-Induced cytidine Deaminase, AID), which is specifically expressed in germinal center B cells of mice (MURAMATSU et al., 1999). This enzyme has been shown to be absolutely necessary for both class switching and somatic hypermutation in mice (MURAMATSU et al., 2000), and mutations in AID are responsible for a variant of the hyper-IgM syndrome in humans (REVY et al., 2000). However, the role of AID in these processes is so far unclear. As the enzyme displays homology to an mRNA-editing enzyme (MURAMATSU et al., 1999), it has been suggested that AID is an RNA-editing enzyme that converts

mRNAs encoding unknown proteins into those of the proteins responsible for DNA breaks during class switching and somatic hypermutation (MURAMATSU et al., 2000). However, even newer findings indicate that AID triggers deamination of dC bases in DNA (PETERSEN-MAHRT et al., 2002), which may give rise to transitions ($dC \rightarrow dT$ and $dG \rightarrow dA$) at the resulting dU bases if they are not repaired (DI NOIA & NEUBERGER, 2002). The finding has also lead to the proposal that the enzyme is responsible for the formation of an initiating DNA break through a deamination mechanism in class switching, somatic hypermutation, and gene conversion (HARRIS et al., 2002; PETERSEN-MAHRT et al., 2002).

3.4 Secondary Rearrangements

A series of secondary rearrangement processes of the antibody V genes have the potential to provide additional antibody diversity. These processes include receptor editing, receptor revision and gene conversion, which, although they share common features, are considered as separate processes.

Receptor editing and revision are two highly related processes, where one of the original V region genes is replaced, entirely or only partly, by a second rearrangement, which in both cases seems to involve RAG activity (NEMAZEE, 2000; NEMAZEE & WEIGERT, 2000; CASELLAS et al., 2001). One key distinction between the two processes is the location where they occur. Receptor editing is believed to take place in the bone marrow in order to make autoreactive immature B cells self tolerant (NEMAZEE, 2000), whereas receptor revision occurs during antigen-driven responses in germinal centers, and is not believed to have a role in immune tolerance (NEMAZEE & WEIGERT, 2000). Most previous studies of secondary rearrangements have been performed with transgenic mice, and have, with a few exceptions (CHEN et al., 1995; LÓPEZ-MACÍAS et al., 1999), dealt with light chain gene replacements. However, a couple of recent studies have identified human B cell clones whose heavy chain V genes have undergone concurrent somatic hypermutation and receptor revision, which has generated hybrid V genes (ITOH et al., 2000; WILSON et al., 2000). This finding indicates a possible second difference between receptor editing and receptor revision. Whereas replacements of light chain genes usually

occur through a second V to J rearrangement, this route is not accessible to heavy chain secondary rearrangements since the D segments are deleted by the first recombination, and VH to JH recombination does not work (NEMAZEE & WEIGERT, 2000). The detected heavy chain replacements have instead been suggested to occur at cryptic heptamer sequences embedded in the IGHV genes, leading to hybrid V genes (ITOH et al., 2000; WILSON et al., 2000). Furthermore, these events have been observed in B cells that express RAG-1, suggesting that these replacements also take place in a RSS-specific mode (ITOH et al., 2000; WILSON et al., 2000).

Gene conversion is another diversification process that produces hybrid V genes. It is characterized by transfer of homologous sequences from a donor antibody V gene segment to an acceptor V gene segment, and it is generally viewed as a non-site-specific homologous recombination event in contrast to the RSS-specific events described above. Gene conversion is an important mechanism for diversification of antibodies in various species, such as chickens, rabbits, and cows, but it is unclear whether it takes place in other species. Selsing *et al*. have, however, demonstrated that sequence transfers resembling gene conversion occur in transgenic mice, and they have also suggested that they may occur in humans (SELSING et al., 1996; D'AVIRRO et al., 2002; TSAI et al., 2002). Furthermore, these studies have also shown that gene-conversion-like events are invariably accompanied by somatic point mutations, which suggests that the same mechanism is involved in both gene conversion and somatic hypermutation. In fact, AID has been implicated as responsible for both processes (HARRIS et al., 2002; PETERSEN-MAHRT et al., 2002).

3.5 Insertions and Deletions in Antibody Variable Domains

Somatic hypermutation was originally thought to only involve single nucleotide substitutions. However, an additional mechanism for the creation of antibody diversity during the somatic hypermutation process has recently been discovered. Several groups have independently discovered insertions and deletions of nucleotides in hypermutated genes encoding antibody variable regions (GOOSSENS et al., 1998; OHLIN & BORREBAECK, 1998; WILSON et al., 1998; DE WILDT et al., 1999b). Insertions and deletions in human V genes have

admittedly been described previously (WU & KAARTINEN, 1995; ANDRIS & CAPRA, 1996), but what distinguishes the novel findings is that the insertions and deletions involve entire codons, which has the potential of giving rise to functional antibodies. In fact, a number of human antibodies with known specificities, carrying this kind of modifications, have been identified (TABLE I). Another distinguishing feature is that most of the insertions and deletions found in V genes isolated from IgG^+ (or IgD^-) B cells are located in or adjacent to the CDR (WILSON et al., 1998; DE WILDT et al., 1999b), suggesting that they have been subjected to antigen selection.

These modifications have been estimated to occur in 1.5-6% of all antibodies produced by normal B cells that have undergone somatic hypermutation, but as insertions and deletions cannot be identified in CDR3 of the heavy chain due to the complexity of the origin of this region and the difficulties associated with the assignment of a germline counterpart, this frequency is likely to be much higher. Limited data indicate that this region is also targeted with modifications of this kind (WILSON et al., 1998). As the most notable difference between genes belonging to the same subgroup is the difference in CDR length (PASCUAL & CAPRA, 1991; LEFRANC & LEFRANC, 2001), it may be argued that the observed sequence modifications are the result of allelic variation among V genes. Sequencing of genomic DNA from donors with insertions and deletions in their antibody genes has, however, shown that the insertions and deletions are truly the result of somatic hypermutation, as no matching germline alleles have been identified (OHLIN & BORREBAECK, 1998; WILSON et al., 1998).

The mechanism responsible for these modifications is not fully understood, but accumulating data from studies with normal B cell populations indicate that they are a direct result of the hypermutation process (GOOSSENS et al., 1998; WILSON et al., 1998; DE WILDT et al., 1999b). First, they are only found in B cells that are or have been part of the germinal center reaction, and they are always accompanied by additional mutations. Second, they seem to accumulate in or around the CDR, which are believed to be the target of the hypermutation and/or selection process (RAJEWSKY, 1996; DÖRNER et al., 1998a).

Antibody	Specificity	Modification	Reference
71-31	HIV-1 p24	1 aa deletion at position 62 in the CDR2-IMGT loop	(ANDRIS et al., 1991)
HBp2	*Bordella Pertussis* lipo-oligosaccharide	1 aa insertion after position 32 in the CDR1-IMGT loop	(ANDRIS et al., 1993)
K14	HIV-1 gp41	1 aa insertion after position 56 in the CDR2-IMGT loop	(VAN DER DONK et al., 1994)
ITC33	HCMV gB	3 aa insertion after position 26 at the beginning of the CDR1-IMGT loop	(OHLIN et al., 1994)
MO53	HCMV pp65	1 aa deletion at position 3 in FR1-IMGT	(RIOUX et al., 1995)

TABLE I. Examples of human antibodies with known specificities that carry amino acid (aa) insertions and deletions in their V regions.

Third, they seem to involve sequence motifs that are considered to be hotspots for the mutational machinery (ROGOZIN & KOLCHANOV, 1992; DUNN-WALTERS et al., 1998; DÖRNER et al., 1998b). Another highly interesting observation is that insertion and deletion events frequently involve duplication of an adjacent sequence or removal of a repetitive part of the sequence. This observation points to the polymerase slippage model by Streisinger *et al*. as a probable mechanism for these modifications (STREISINGER et al., 1966; RIPLEY, 1990). This model postulates that the DNA strand that is being synthesized becomes misaligned as the polymerase slips, and anneals to an adjacent repetitive stretch on the other strand. The misaligned DNA forms an unpaired loop between the repeats and will lead to an insertion or a deletion, depending on which strand the loop is located on, if it is not repaired by the proofreading machinery (FIG. 2). As the generation of insertions and deletions require strand breaks at some point or other, the mechanism of DNA double strand breaks has also been implicated as responsible for these modifications (GOOSSENS et al., 1998; BROSS et al., 2000). It is likely that both mechanisms cooperate in the generation of insertions and deletions. Unpaired loops may initially be formed by polymerase slippage and subsequently converted to insertions or deletions by a process that involves DNA strand breaks.

This possible involvement of repetitive nucleotide sequences in antibody variable genes in the process of single-codon deletions was investigated by us in PAPER I. We analyzed the human germline V gene repertoires for the occurrence of trinucleotide repeats by a method, whereby each nucleotide along a sequence was assigned a score depending on the similarity of the trinucleotide it is located in to the preceding trinucleotide (PAPER I: see legend to Fig. 2). This study was focused on trinucleotide repeats since these are more likely to lead to in-frame insertions and deletions due to the addition or removal of an entire codon by the proposed polymerase slippage mechanism (see FIG. 2). Furthermore, single-codon deletions can easily be reconciled with this mechanism of nucleotide insertion or deletion, as they will probably involve rapid reannealing to an adjacent repetitive sequence, thereby eliminating a time-consuming search through a large space for a suitable sequence to anneal to.

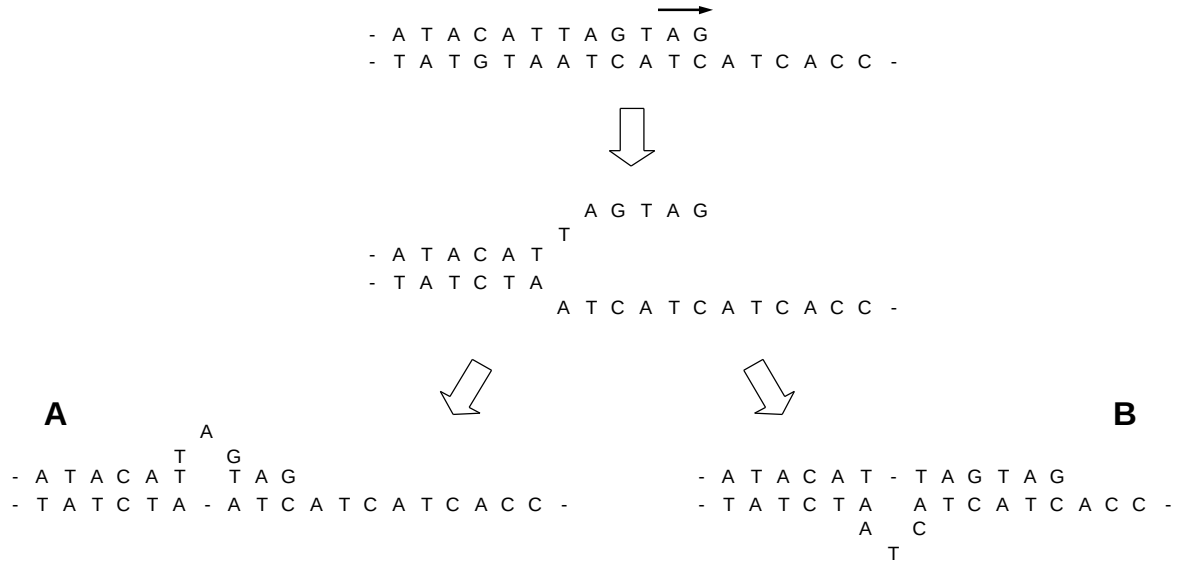

FIG. 2. Creation of a single-codon insertion or deletion during DNA replication according to the polymerase slippage model as first suggested by Streisinger and colleagues (STREISINGER et al., 1966). In a stretch of trinucleotide repeats, the polymerase slips or stutters, and the strand that is being synthesized becomes misaligned. Upon reannealing of the two strands, a short unpaired loop is created, which will lead to an insertion if it is located on the daughter strand (A), or a deletion if it is located on the template strand (B). The solid arrow indicates the direction of synthesis.

By analyzing the human IGHV repertoire in this manner, we were able to show that many germline genes carry accumulations of repetitive elements, in particular in and around CDR1 and CDR2. Heavy chain variable genes are in general more mutated than light chain genes (TOMLINSON et al., 1996; DE WILDT et al., 1999a), and it is possible that this is also the reason why fewer insertions and deletions have been observed in light chain variable genes (GOOSSENS et al., 1998; DE WILDT et al., 1999b). However, our analysis showed that the IGKV and IGLV germline gene repertoires do not display as pronounced accumulations of repeats, except for in a few cases (PAPER I: supplementary data), which may provide an additional explanation to the low frequency of such modifications. In line with these observations, an analysis of single-codon deletions from the literature showed that an absolute majority of these events have occurred in regions containing trinucleotide repeats. This strongly indicates

that repetitive sequence elements are indeed targeted with deletions during the somatic hypermutation process. Furthermore, the observed patterns of trinucleotide repeats suggest that the CDR are particularly predisposed to undergo such modifications.

The observed patterns of trinucleotide repeats may also provide further insights into the evolution of the V gene repertoires. It is commonly believed that the IGHV families arose by duplication of an ancestral gene, followed by further diversification, mainly of the CDR. The differences between the germline genes have been shown to include not only amino acid substitutions, but also insertions and deletions that have altered the lengths of the CDR (PASCUAL & CAPRA, 1991; ROTHENFLUH et al., 1994; 1995). The concentration of trinucleotide repeats in the CDR that we found in PAPER I suggests that the different CDR lengths of the IGHV genes have evolved by duplication of adjacent trinucleotides. While increasing the potential structural repertoire encoded by the evolving V genes, this process has also ensured that the transcripts have remained in frame, and therefore also likely capable of producing functional proteins. Indeed, it has been proposed that antibody V genes are under some form of selection pressure based on protein function (PASCUAL & CAPRA, 1991; ROTHENFLUH et al., 1995). Furthermore, it has been hypothesized that the diversity of canonical loop structures in the germline repertoire has evolved concurrently with the evolution of the V genes by this process of codon insertion. This evolutionary process has apparently also been subject to a selection pressure, as the insertions in both human and murine germline V genes have occurred at positions in the CDR that allow for the preservation of the key residues that define the canonical structures (ALMAGRO et al., 1997; VARGAS-MADRAZO et al., 1997).

A closer inspection of the accumulation of trinucleotide repeats in genes belonging to the large IGHV3 and IGHV4 subgroups showed that these subgroups apparently have evolved their CDR2 independently. The foci of the repetitive sequence elements in CDR2 of genes from the two subgroups differ by 15-20 bases (PAPER I: Fig. 3), suggesting that the insertion of codon(s) has been targeted to different locations during the evolution of these IGHV subgroups. The different locations of the repetitive tracts have implications for

the somatic diversification of antibodies as well, as it seems that naturally occurring insertions and deletions are differently targeted, in accordance with the distribution of trinucleotide repeats, in genes belonging to either subgroup (see, for instance, PAPER I: Fig. 1). It has been shown that germline gene-encoded diversity is focused at the center of the antigen-binding site in both the heavy and light V gene repertoires, whereas somatic hypermutation spreads diversity to peripheral regions that are highly conserved in these repertoires (TOMLINSON et al., 1996; IGNATOVICH et al., 1997). The location of the repetitive sequence elements in CDR2 of genes belonging to the IGHV4 subgroup suggests that deletions in such genes will mainly be targeted to regions in the periphery of the antigen-binding site that are relatively conserved in the germline repertoire, but are frequently targeted by somatic mutation. IGHV3-derived genes, on the other hand, are more likely to have such modifications introduced close to the center of the binding site. It is possible that the pattern of trinucleotide repeats is simply a consequence of the differential targeting of insertions during the evolution of the germline genes, and that it, due to the relative rarity of insertions and deletions, has not had an impact on the selection of individual genes or subgroups. Alternatively, the differences in location of trinucleotide repeats may have a functional meaning.

The combinations of loop lengths (structures) encoded by the investigated genes have been associated with multi-specific gross topographies of the antigen-binding site (VARGAS-MADRAZO et al., 1995), indicating that a number of different types of antigens may be recognized by the products of these genes. Naturally, the gross topographies are also dependent on the pairing of the heavy chains with light chains carrying the appropriate loop structures. However, since it is apparent that large antigens make more contacts with residues further away from the center of the binding-site than smaller antigens, as judged by the large difference in buried surface area between different antibody-antigen complexes (WILSON & STANFIELD, 1993; WEBSTER et al., 1994; BRADEN & POLJAK, 1995; DOKURNO et al., 1998), the location of the repetitive regions may indicate that the investigated members of the two IGHV subgroups are evolutionary predisposed to be able to fine-tune the response against different types of antigens.

Due to our finding that functional insertions and deletions seem to be targeted to the CDR of human antibodies, and the fact that this process may expand the repertoire of CDR loop lengths and conformations, we decided to investigate the functional consequences of such modifications in PAPER II. As shown in TABLE I, the number of human antibodies with known specificities that carry modifications of this kind is very limited, and although insertions have been used to engineer murine antibody specificities (LAMMINMÄKI et al., 1999; PARHAMI-SEREN et al., 2002), relatively little is known about the functional consequences of such alterations of antibody sequence and structure space. We therefore created single-codon insertions and deletions in CDRH1 and CDRH2 of human antibody fragments of known specificities by molecular engineering and characterized the resulting modified scFv variants in terms of protein folding, stability, and antigen recognition.

The modifications were introduced in regions of the molecules that we in PAPER I found carried repeated sequences and thus were naturally targeted by such events, and that also are known to frequently make contact with the antigen in antibodies of other specificities (MACCALLUM et al., 1996). These scFv variants were cloned into the methylotrophic yeast *Pichia pastoris* as described in PAPER III and screened for antigen binding or production of soluble scFv. Subsequent characterization of the different variants showed that a wide range of amino acids were tolerated at the different positions in both CDR, as judged by the retained antigen specificity, which indicated that the immunoglobulin fold of the variant scFv was intact. Stability measurements further confirmed that the modifications did not affect the integrity of the immunoglobulin fold of the altered scFv clones.

As the single-codon modifications were introduced at the apices of the CDR loops, the positions where the natural length variation within the germline gene repertoire is believed to have occurred, or at least has manifested itself (CHOTHIA et al., 1992; VARGAS-MADRAZO et al., 1997), it is perhaps not surprising that they were so well tolerated in general. However, by analyzing the possible canonical structures of the modified loops, a number of interesting observations were made. First of all, the single-codon insertions created loop lengths that do not occur normally within the human IGHV3 subgroup, which

both of the studied scFv are derived from, and they also gave rise to combinations of loop lengths that do not exist in the human germline repertoire. As mentioned previously, both the set of canonical structures and the combinations of structures are limited (VARGAS-MADRAZO et al., 1995; AL-LAZIKANI et al., 1997), which has been suggested to be indications of restrictions acting on the diversification of the loop structures and their combination within the same antibody (ALMAGRO et al., 1998). However, the obtained results, and the fact the same combinations have been observed in seemingly functional hypermutated antibodies with insertions in CDRH1 (DE WILDT et al., 1999b), suggest that the potential restrictions are not very rigid. Furthermore, the structure classification also revealed that a large number of the key residue requirements for the canonical structure that best corresponded to the created CDRH1 loop length were not fulfilled (MARTIN & THORNTON, 1996).

This may indicate that there is larger flexibility in the key residue requirements for a particular loop structure than previously believed, or it may possibly indicate that entirely new structures were created by the insertions. In fact, it was recently demonstrated that camelid heavy-chain antibodies (antibodies that lack light chains), which are highly similar to human VH domains in sequence and structure, carry CDR loops that do not fit the current set of canonical loop structures (DECANNIERE et al., 2000). Furthermore, the single-codon modifications of antibody sequence space that were created in PAPER II seem to be highly representative of changes that may occur naturally in the human antibody repertoire as a consequence of the somatic hypermutation process. Apparently functional human antibodies belonging to the IGHV3 subgroup with insertions and deletions in CDRH1 and CDRH2, at or immediately adjacent to the positions targeted in this study, leading to the same loop lengths, have relatively frequently been encountered (BREZINSCHEK et al., 1997; DE WILDT et al., 1999b; NOPPE et al., 1999; and PAPER I: Fig 1). As new structures may arise in human as well as in other vertebrate antibodies, it seems thus as though the set of canonical loop structures suggested by Chothia *et al.* (CHOTHIA et al., 1992; AL-LAZIKANI et al., 1997) is incomplete.

The results from PAPER II demonstrate the plasticity of antibody V domain frameworks belonging to the important IGHV3 subgroup and its capacity to tolerate single-codon modifications that expand sequence and structure space beyond the limits set by the germline-encoded diversity. As some of the single-codon insertions produced loop lengths found in antibodies belonging to the IGHV4 subgroup, we set out to investigate the possibility of using CDRH1 sequences originating from this subgroup to diversify an antibody fragment originating from the IGHV3 subgroup. This would create hybrid antibody V genes in a manner resembling natural diversification through receptor revision (ITOH et al., 2000; WILSON et al., 2000), or perhaps even more gene conversion (D'AVIRRO et al., 2002; TSAI et al., 2002), although the latter has not been definitely demonstrated in humans. In fact, the process of receptor revision has been shown to allow for the combining of IGHV genes from different subgroups (ITOH et al., 2000). However, grafting of CDRH1 loops of different lengths and, presumably, canonical structures from the IGHV4 subgroup (PAPER II: Table II) into the IGHV3 framework used in this study did not result in a productive diversification of the sequence and structure space, as the modifications resulted in a polyreactive character in all of the tested variant clones.

Analysis of the secondary structure of two of the modified clones demonstrated an absence of the β-sheet conformation that is characteristic of antibody domains (PAPER II: Fig. 4) (POLJAK et al., 1973; AMZEL & POLJAK, 1979; PADLAN, 1994; WOODY, 1995), which indicated that the V domains of these clones were inappropriately folded. This lack of organized secondary structure probably explains the polyreactivity of the loop-grafted clones, but it remains to be established exactly why these clones did not fold properly. One possible reason for the observed behavior can be found in the key residues that are used to define canonical loop structures (CHOTHIA et al., 1992; MARTIN & THORNTON, 1996). Examination of amino acid sequences encoded by V genes belonging to the IGHV3 and IGHV4 subgroups showed that they differed at key residues in the frameworks. Framework residue 80 was particularly implicated by this analysis, as the two subgroups tend to have highly different amino acids at this position. Antibodies originating from the IGHV3 subgroup, including the one used in the study, usually carry an arginine, which is a bulky, charged amino

acid, at this position. IGHV4-derived antibodies, on the other hand, usually have a valine, which is relatively small and nonpolar. It has been shown that this residue packs against residues in both CDRH1 and CDRH2, and that it is an important determinant of the conformation of the CDRH2 loop and thus the binding characteristics of an antibody (TRAMONTANO et al., 1990; XIANG et al., 1995). The improper folding of the loop-grafted clones may be due to clashes between the arginine residue of the IGHV3-derived framework and the IGHV4-derived residues in and adjacent to the grafted CDRH1 loop.

This finding may have implications for the possibility of the human heavy chain repertoire to diversify through processes that create hybrid V genes and for the feasibility of using loop grafting in antibody engineering. It seems likely that only those secondary rearrangements (or recombinations) that preserve important key residues will lead to functional antibodies. CDR loop grafting has been utilized for the creation of large human antibody scFv libraries, and the CDRH1 and CDRH2 carried by the variants selected from these libraries against a number of different antigens have invariably been derived from the same subgroups as the frameworks (SÖDERLIND et al., 2000; JIRHOLT et al., 2001; 2002; ELLMARK et al., 2002a; 2002b; and PAPER III). Another example where these restrictions may have an impact is in antibody humanization (reviewed in DALL'ACQUA & CARTER, 1998). This is the process of grafting CDR loops from rodent antibodies into a human antibody framework in order to avoid the immune response evoked by the rodent antibodies. Successful humanization has been reported in a large number of cases, but it has also been noted that it is necessary to incorporate a number of key residues from the rodent antibody in the human framework in order to obtain functional antibodies. Thus, it seems that in most cases, attention must be paid to key residues in the framework in order to achieve success with loop grafting.

4 ANTIBODY REPERTOIRE DEVELOPMENT

4.1 The Germline Gene Repertoire

The complete repertoires of human immunoglobulin germline gene segments have recently been mapped and sequenced (see, for instance, TOMLINSON et al., 1992; ZACHAU, 1993; WILLIAMS et al., 1996; CORBETT et al., 1997; MATSUDA et al., 1998), and all this information has been made available in a database, IMGT, the international ImMunoGeneTics database (LEFRANC & LEFRANC, 2001). It is now possible to more precisely estimate the number of functional genes that participate in the process of rearranging antibody V genes and thus shape the repertoire of specificities that invading pathogens encounter. According to IMGT, the potential IGH repertoire consists of 38-46 functional IGHV genes, depending on allelic polymorphisms and uncertainties about the functionality of alleles, which can be divided into seven subgroups based on sequence homology. In addition, there are 23 functional IGHD genes and 6 functional IGHJ genes. The corresponding numbers for the light chain gene repertoires are 31-35 functional IGKV genes belonging to five subgroups, 29-33 functional IGLV genes belonging to ten subgroups, and five and four IGKJ genes and IGLJ genes, respectively (LEFRANC & LEFRANC, 2001).

Within each germline gene repertoire, the different subgroups contain different numbers of functional genes. In the IGHV repertoire, the IGHV3 subgroup contains approximately half of the functional genes and is by far the largest subgroup. In the light chain V gene repertoires, the IGKV1 and IGLV3 subgroups are the largest, and constitute approximately one half and one third of the functional repertoires, respectively (LEFRANC & LEFRANC, 2001).

4.2 Natural Antibodies

This somewhat restricted repertoire of V, D, and J segments puts limits on the size of the possible repertoire, which is difficult to correlate to the numerous structures recognized by antibodies. Usually, the wide array of specificities encoded by the human antibody repertoire is attributed to the combinatorial and hypermutational processes that diversify the repertoire (see chapter 3), but this view has recently been challenged by Rao and colleagues (MANIVEL et al.,

2002). They found that germline gene-encoded antibodies display a great deal of heterogeneity in their recognition of random peptides, whereas related, affinity matured antibodies do not. Furthermore, they were able to show that this promiscuity of the germline antibodies is a consequence of antigen-binding site plasticity, which apparently allows for an expanded recognition repertoire of each germline antibody. It is thus possible that this feature enables the rather limited germline repertoire to encompass the multitude of antigenic determinants that the immune system has to deal with, and, in addition, it ensures a rapid induction of a primary response that can be further evolved to fit each specific antigen (MANIVEL et al., 2002). The notion that antigen-binding sites may display plasticity, or conformational isomerism, leading to an increased diversity and recognition potential of the antibody repertoire, is also supported by the work of others (FOOTE & MILSTEIN, 1994).

The results from the study by Manivel *et al*. resemble what has previously been observed with so-called natural antibodies. These are immunoglobulins that are present in the serum of vertebrates without deliberate immunization and apparently independent of exposure to foreign antigen (AVRAMEAS, 1991; COUTINHO et al., 1995). They are encoded by germline genes carrying no, or few mutations, and are of the IgM, IgG, and IgA isotypes. Most natural antibodies are multireactive and bind to evolutionary conserved antigens (including auto-antigens) with low affinity. However, it is a consistent finding that each natural antibody has its own range of specificities (COUTINHO et al., 1995). The origin of the stimuli that is responsible for their generation is still being debated, but contact with non-pathogenic microorganisms, intestinal bacterial flora, and food, have been suggested to be the sources of the antigenic challenge. However, the presence of natural antibodies in germfree and antigen-free animals suggests a role for self antigens in the generation of these multireactive antibodies (HAURY et al., 1997). The primary physiological function of natural antibodies seems to be in the first line of defense against infections, together with components of the innate immunity. They are also believed to be involved in the protection against many viruses (BOES, 2000; ZINKERNAGEL et al., 2001).

4.3 Specific Antibody Repertoires

The dominance of certain subgroups in the germline gene repertoires is also reflected in repertoires of rearranged antibodies, as has been shown in a number of studies. However, the same studies have also indicated that the V gene usage is not entirely random. In the heavy chain repertoire in normal B cells, the IGHV3 subgroup is most frequently used, largely due to the preferential usage of a few genes such as IGHV3-23 and 3-30, followed by the IGHV1 and IGHV4 subgroups (HUANG et al., 1996; BREZINSCHEK et al., 1997; DE WILDT et al., 1999a). The remaining subgroups account for only about 10% percent of the total heavy chain repertoire. Although complicated by the existence of two different light chain isotypes, the picture is similar regarding the light chain repertoire, with the IGKV1 and IGKV3 subgroups accounting for approximately half of the IgG rearrangements, and the IGKV3-20, IGKV1-39/IGKV1D-39, and IGKV3-15 being the most frequently expressed genes (COX et al., 1994; IGNATOVICH et al., 1997; DE WILDT et al., 1999a). Furthermore, the heavy and light chain pairing seems to be random as the most frequently used genes also tend to be paired in functional IgG antibodies, with an IGKV to IGLV ratio of 60:40 (DE WILDT et al., 1999a; BENGTÉN et al., 2000). The predominant usage of certain IGHV genes has been shown to be determined to a significant degree by their preferential rearrangement during V(D)J recombination (RAO et al., 1999). Similar biases have been observed in the IGKV repertoire, and it has been suggested that this preferential rearrangement of some genes is linked to the chromosomal location of individual genes or differences in the RSS (COX et al., 1994; FEENEY et al., 2000).

In the majority of the responses against exogenous antigens, there seems to be no restrictions in the usage of particular V region genes (ANDRIS & CAPRA, 1996; OHLIN & BORREBAECK, 1996). However, many reports on antibody responses have dealt with poorly defined antigens, and it is possible that repertoires reactive with particular epitopes on an antigen are indeed restricted in terms of V gene usage. In fact, such biases have been observed in many different antibody responses against defined epitopes. This was initially demonstrated in murine antibody responses against haptens like phenyl-oxazolone (MÄKELA et al., 1978; KAARTINEN et al., 1983; BEREK & MILSTEIN,

1987), p-azophenylarsonate (MÄKELA et al., 1976; MANSER, 1990), and 4-hydroxy-3-nitrophenyl)acetyl (IMANISHI & MÄKELA, 1974; 1975; BLIER & BOTHWELL, 1988), which all constitute a single, well-defined epitope. Similarly, human antibody responses against *Haemophilus influenzae* type b polysaccharide have been shown to be restricted to genes originating from the IGHV3 subgroup (SILVERMAN & LUCAS, 1991; ADDERSON et al., 1993; LUCAS & REASON, 1999). Recently it has also become clear that particular variable region genes dominate the antibody response against a multitude of proteins and other complex antigens. These include both pathogens, allergens, and self antigens. For example, human and murine antibody repertoires against single epitopes on lysozyme (GOLDBAUM et al., 1999; LAVOIE et al., 1999), HIV-1 (BINLEY et al., 1996), *Neisseria meningitidis* (IHLE & MICHAELSEN, 2001), rabies virus (IKEMATSU et al., 1998), the Pfs48/45 protein of *Plasmodium falciparum* (ROEFFEN et al., 2001), peanut allergen (JANEZIC et al., 1998), trichosantin (WANG & YEH, 1996), house dust mite (SNOW et al., 1995), timothy grass pollen allergen (STEINBERGER et al., 1996), the C2 domain on factor VIII (VAN DEN BRINK et al., 2000), and different components of the U1 small nuclear ribonucleoprotein particle (DE WILDT et al., 1996; HOET et al., 1998; DEGEN et al., 2000) have all been shown to display restrictions in V region gene usage. Accumulating evidence suggests thus that intrinsic features of individual epitopes restrict the V gene usage of the responding repertoire.

What are the forces that influence the usage of specific V region genes in an immune response against a particular epitope? Most immunologists agree that the germline repertoire of antibody V genes has evolved under the selection pressure exerted by pathogenic microorganisms. However, opinions differ as to how this evolution has occurred. Cohn and Langman have suggested that individual genes have been maintained in the germline repertoire due to their capacity to recognize antigens on pathogens, which the elimination of are of great importance for the survival of the species (COHN & LANGMAN, 1990). Although a plausible explanation to the generation of the germline gene repertoire, it has been argued that investing specificity for a pathogen into one single gene puts the individual at risk of losing that gene, and thereby also the protection against this particular pathogen (LUCAS & REASON, 1999). Instead, it

has been suggested that the key factor determining if a gene is retained in the germline repertoire or not is its capacity to contribute to diversity by being a suitable template for further somatic diversification (COLECLOUGH, 1990; LUCAS & REASON, 1999). Despite this apparent drawback of investing specificity into individual genes, the immune repertoire may still have an imprint of V genes that are required to recognize certain targets for which there is a great survival advantage to respond rapidly against. For instance, it has been shown that high-affinity, neutralizing murine antibodies against vesicular stomatitis virus, a cytopathic virus, are germline encoded (ROOST et al., 1995; KALINKE et al., 1996). Similarly, the protective antibody response to *Streptococcus pneumoniae* is provided by germline-encoded phosphocholine-specific antibodies (CLAFLIN & BERRY, 1988). In neither case do somatic mutations that are introduced during the secondary response provide but marginal improvements in antigen binding (CLAFLIN & BERRY, 1988; KALINKE et al., 1996). However, it has been suggested that, since vesicular stomatitis virus is not a natural mouse pathogen, it is the virus that has evolved to fit the vertebrate antibody repertoire in general rather than the other way round (ROOST et al., 1995; ZINKERNAGEL et al., 2001).

If we for a moment accept the idea of an antibody V gene repertoire that has evolved in response to a variety of antigenic determinants displayed by pathogens, it stands clear that very few antigens have had a selective impact on the repertoire given the limited number of available V genes. However, every antigen will be faced with the same naïve antibody repertoire having these few basic structures. As the initiation and maturation of a B cell response requires an affinity for the antigen above a certain threshold (BATISTA & NEUBERGER, 1998; 2000), the products of V genes that carry intrinsic features, which allow them to provide critical contacts with the antigen, may have an advantage in the selection process. Based on studies in mice, it has been suggested that there does not exist an intrinsic affinity threshold for B cell entry and survival within germinal centers, as B cell clones carrying receptors of both low and high affinity can be retrieved from germinal centers within the same spleen (VORA & MANSER, 1995; DAL PORTO et al., 1998). Furthermore, the results also indicate

that affinity maturation in germinal centers is local, i.e. there is no competition between germinal centers. However, another study has shown that when both high- and low-affinity B cells are transferred into mice, only the high-affinity clones will proceed beyond the very earliest stages of the germinal center reaction (SHIH et al., 2002). B cell receptor triggering *in vivo* most likely requires the extraction of immobilized antigen from antigen presenting cells, a process that is dependent on B cell receptor affinity for the antigen over a wide affinity range (BATISTA & NEUBERGER, 2000). It is possible that the low affinity clones cannot compete for the available antigen with B cell clones that carry receptors with an imprinted high affinity for the antigen and a capacity to extract and internalize the antigen more rapidly. The high-affinity clones may thus come to dominate the repertoire by eliminating the antigen that is required to sustain the other, initially existing, low-affinity clones, and will consequently direct the immune response towards structures on the antigen against which they are optimally suited.

Xu and Davis have demonstrated that transgenic mice having access to only a single human IGHV gene (IGHV5-51) and three murine IGLV genes were able to mount antibody responses against a number of protein antigens and haptens (XU & DAVIS, 2000). Furthermore, upon rechallenge, the antibodies were able to improve the affinity of the interaction with the antigen by somatic hypermutation. Although generally considered as highly immunogenic, bacterial polysaccharides were the only antigens that did not evoke a response (XU & DAVIS, 2000). The results from this study suggests that any one germline V gene may form the template for an antibody response against essentially any complex antigen as long as there is sufficient diversity in CDRH3. However, it cannot be excluded that certain epitopes cannot interact effectively with a particular single V gene antibody repertoire, as evidenced by the lack of antibodies against the bacterial polysaccharides (XU & DAVIS, 2000). Furthermore, as noted above, in a natural situation B cell clones will compete for dominance and survival based

on a number of factors like frequency in the repertoire, and reaction rate and affinity of the interaction with the antigen. For instance, the recurrent usage of particular VH genes in the response against p-azophenylarsonate has been suggested to be due not only to antigenic selection, but also to the high probability with which these VH genes are formed during B cell differentiation (MANSER, 1990).

Interestingly, restrictions in V gene usage may have nothing to do with the binding to the epitope. It has been noted that antibodies against the Rh(D) antigen of the Rhesus blood groups preferentially use some V gene segments, which upon closer inspection have been shown to be some the most cationic in the human IGHV repertoire (BOUCHER et al., 1997). The finding that the cationic residues in the products of these V genes are not clustered in the CDR indicated that the functional role of cationic restriction may not be related to the binding to Rh(D) epitopes. Rather, it has been suggested that the positive charge of the antibodies possibly facilitates contact with the antigen in the negatively charged membrane environment of red blood cells (BOUCHER et al., 1997). A similar observation has also been made with repertoires of autoantibodies reactive with platelets, another cell type that has a very highly negatively charged surface (ROARK et al., 2002).

5 ANTIBODIES AGAINST HUMAN CYTOMEGALOVIRUS

In addition to the examples in the previous chapter, human cytomegalovirus (HCMV) is another pathogen that may give rise to restricted antibody responses. Antibodies against neutralization epitopes on an abundant membrane glycoprotein display a V gene usage that suggests that intrinsic features of the germline gene repertoire and/or the epitopes influence the response against this antigen.

5.1 Human Cytomegalovirus

HCMV is a member of the *Herpesviridae* that has a high prevalence in the population, but causes little or no harm to healthy individuals. However, the virus constitutes a serious threat to immunocompromized patients, such as transplant recipients and AIDS patients, as well as to newborns. In fact, HCMV is probably the major infectious cause of birth defects in developed countries. Infection with HCMV may cause a variety of clinical syndromes, including prolonged fevers, mononucleosis, pneumonitis, and retinitis (HO, 1995; BRITT & ALFORD, 1996). Individuals with a functional, mature immune system mount a strong immune response that suppresses persistent viral replication, but as the virus has evolved a multitude of ways of modulating the host immune response (LOENEN et al., 2001), the immune system is prevented from eliminating the virus completely, thus allowing it to establish life-long latency. Loss of immune control, such as during immunosuppressive treatment, opens the way to virus reactivation and possibly severe disease.

The large number of evasive strategies employed by HCMV involves modulation of both the innate and adaptive arms of the immune response, but appear to target cellular rather than humoral responses preferentially. The virus encodes a diverse arsenal of proteins that interfere with many processes, including activation of MHC-restricted T and NK cells, leukocyte migration, and cytokine responses (MICHELSON, 1999; LOENEN et al., 2001).

The immunological effector functions that control HCMV infections are currently poorly understood. Whereas cellular immunity seems to be crucial for resolving the infection, accumulating evidence suggests that humoral immunity has an important role in protecting against the virus as well. It is rather well

established that the presence of maternal antibody to CMV prior to conception will provide a substantial protection against harmful HCMV disease in the newborn (YEAGER et al., 1981; TANAKA et al., 1991; FOWLER et al., 1992; ADLER et al., 1995). Passive transfer of anti-HCMV antibodies has also been shown to improve the outcome of the infection in both transplant patients and prematurely born infants (CONDIE & O'REILLY, 1984; WINSTON et al., 1987; ZAIA, 1993; SNYDMAN et al., 1995). In addition, the level of neutralizing anti-HCMV antibodies in the blood is inversely related to the systemic viral load in both transplant recipients and AIDS patients (SCHOPPEL et al., 1997; ALBEROLA et al., 1998). Furthermore, in the closely related murine cytomegalovirus system, it has been shown that adoptive transfer of cytomegalovirus-specific antibodies provide protection against fatal disease (FARRELL & SHELLAM, 1991), and that antibodies are also responsible for limiting virus spread during virus reactivation (JONJIC et al., 1994).

For a long time, polyclonal antibody preparations have been used to prevent HCMV disease, although with conflicting results (BRITT & ALFORD, 1996). One reason for these inconsistencies may be that the composition of such preparations is variable and usually of low titre (SCHMITZ & ESSUMAN, 1986; CHEHIMI et al., 1987; ROY & GRUNDY, 1992), precluding an efficient and reproducible utilization of them. However, several *in vitro* studies have demonstrated neutralization of HCMV infectivity using both murine and human monoclonal antibodies directed against different epitopes on the virions (reviewed in SPAETE et al., 1994; BRITT & MACH, 1996). For that reason, clinical trials have been initiated, which, although inconclusive regarding the beneficial effects of the therapy, have at least demonstrated the safety of specific monoclonal antibodies that would not suffer from inconsistencies found with polyclonal antibody preparations (AZUMA et al., 1991; BOECKH et al., 2001). Similarly, clinical trials addressing the immune responses evoked by various experimental vaccines ranging from live, attenuated vaccines to recombinant subunit vaccines and canarypox virus expressing HCMV genes have been assessed in efforts trying to develop an efficacious vaccine that possibly would be able to induce protective humoral immunity (PLOTKIN, 2001). Particular attention has been given to one of the HCMV envelope proteins, glycoprotein B

(gB), which for several reasons, as outlined below, is an attractive candidate for a recombinant vaccine. Results from trials with a recombinant gB subunit vaccine indicate that this antigen induces high levels of neutralizing antibodies, but also that the response declines with time (FREY et al., 1999; PASS et al., 1999). Furthermore, sera from vaccinated individuals have the capacity to neutralize a range of viral strains and isolates, indicating that the subunit vaccine evokes a broad spectrum of antibodies (PLOTKIN, 2002).

5.2 Glycoprotein B

Antibodies can be raised in both humans and mice against a number of proteins on the virus, including envelope glycoproteins (SPAETE et al., 1994). Among these proteins, gB is particularly interesting for several reasons. Glycoprotein B is the most abundant constituent of the HCMV envelope (BRITT & MACH, 1996) and it has been shown to be involved in many steps of the infection process, such as membrane penetration, cell-to-cell spread, and syncytium formation (NAVARRO et al., 1993; TUGIZOV et al., 1994; BOLD et al., 1996). It is also highly immunogenic in humans and constitutes the major target for neutralizing antibodies (BRITT & MACH, 1996). Moreover, it has the capacity of binding heparin, which suggests that it also plays a role in the initial adhesion to the cell surface, as virus attachment to cell surface heparan sulphate proteoglycans is the first step in the infection process (COMPTON et al., 1993; KARI & GEHRZ, 1993). Recently, it has also been revealed that one of the consequences of the interaction of gB with its yet undefined cellular receptor is the initiation of intracellular signaling and activation of the interferon-responsive pathway (BOYLE et al., 1999; SIMMEN et al., 2001). Although interferons are generally considered to have an antiviral activity, it has been suggested that HCMV has adapted this cellular signaling and gene activation pathway for its own benefit as interferons are required for cellular differentiation and viral replication during HCMV reactivation (BOYLE et al., 1999).

Britt and coworkers have investigated the synthesis and processing of gB extensively, and have been able to show that the mature form of gB is arrived at via a number of modifications of the original approximately 900 amino acids long polypeptide encoded by the gB gene (reviewed in BRITT & MACH, 1996).

The first step is the cotranslational addition of both simple and complex N-linked sugars to this polypeptide to form the 150 kDa precursor that constitutes the main intracellular form of gB (BRITT & VUGLER, 1989). Next, this precursor is further processed into an approximately 170 kDa intermediate that is proteolytically cleaved into an N-terminal (gp116) and a C-terminal fragment (gp55), which together form disulfide-linked complexes (BRITT & VUGLER, 1989; BRITT & MACH, 1996). The final, mature form of gB that can be found in the membranes of infected cells and the envelopes of virions is a homodimer of gp55-gp116 complexes (BRITT & VUGLER, 1989; 1992).

5.3 Antibodies against Antigenic Domains on Glycoprotein B

A number of continuous and assembled epitopes that induce both neutralizing and non-neutralizing antibodies in humans as well as mice have been identified in both fragments of gB (SPAETE et al., 1994). However, two of these, antigenic domains 1 and 2 (AD-1 and AD-2, respectively), have been studied more extensively than others. AD-1 is located on the gp55 subunit of gB and comprises a linear region of more than 75 amino acids (UTZ et al., 1989; WAGNER et al., 1992). AD-2 is located on the gp116 subunit and was originally defined using a monoclonal antibody, C23 (MASUHO et al., 1987; MEYER et al., 1990). The recognition site of this monoclonal antibody was initially mapped between residues 27-84 of gB (MEYER et al., 1990), but it was subsequently shown that this region actually consists of two antibody-binding sites, site I between residues 68-77 and site II between residues 50-54 (MEYER et al., 1992). C23 recognizes the epitope between residues 68-77 (site I) (MEYER et al., 1992), which has also been shown to be the target of the only other published human monoclonal antibody against this part of gB, ITC88 (OHLIN et al., 1993).

AD-1 is the immunodominant region of gB, and it has been demonstrated that the development of antibodies against this structure is nearly universal in infected individuals (SCHOPPEL et al., 1997). In contrast, site I of AD-2 is only weakly immunogenic *in vivo*, and antibodies against this epitope can be detected only in a minority of individuals infected with HCMV (MEYER et al., 1992; AYATA et al., 1994; NAVARRO et al., 1997; OHLIN et al., 1997; SCHOPPEL et al., 1997). Besides the two human antibodies mentioned above, only two murine

monoclonal antibodies recognizing this site have been reported (KARI et al., 1986; CURTSINGER et al., 1994). As antibodies develop efficiently against AD-1 in most seropositive individuals, the poor response against AD-2 does not seem to be related to insufficient amounts of gB present after infection, or the lack of T cell help. However, it has been noticed that the heavy chains of human antibodies specific for AD-1 are frequently encoded by the IGHV5-51 gene (OHLIN et al., 1994), which is the only mapped functional gene of the IGHV5 subgroup (LEFRANC & LEFRANC, 2001). Although these IGHV5-51-encoded heavy chains also contain a number of somatic mutations, including a three-codon insertion at the beginning of CDRH1 in one them (OHLIN et al., 1994; OHLIN & BORREBAECK, 1998; and TABLE I), this restricted V gene usage indicates that the germline repertoire may have an imprinted specificity for AD-1, which can act as a suitable template for the generation of high-affinity antibodies by somatic hypermutation. It is possible that this imprinted specificity for AD-1 enables B cell clones specific for this epitope to outcompete clones that are only weakly reactive with other antigenic determinants, such as AD-2.

Despite the poor immunogenicity of site I of AD-2 in both humans and mice, it occasionally seems to have the capacity to evoke potent, complement-independent neutralizing antibodies. In fact, all four presently known monoclonal antibodies reactive with the epitope neutralize virus in a complement-independent fashion (MASUHO et al., 1987; KARI et al., 1990; OHLIN et al., 1993; FUREBRING et al., 2002), whereas the AD-1-specific antibodies vary in their requirement for complement (SPAETE et al., 1994). As this epitope is also highly conserved among different HCMV isolates (CHOU & DENNISON, 1991; LEHNER et al., 1991; ROY et al., 1993; SHIU et al., 1994; MEYER-KÖNIG et al., 1998), an increased targeting of it, for instance during the response to HCMV vaccination, would potentially be of great value. Currently available data about the immune response in gB subunit recipients demonstrate the induction of antibodies reactive with a recombinant gB protein containing AD-1 and AD-2, but state nothing about the levels of antibodies against the individual epitopes (MARSHALL et al., 2000). However, there is no reason to believe that the recombinant subunit vaccine will evoke significantly higher levels of antibodies against site I of AD-2 than natural infection does.

In order to investigate the basis for the poor development of antibodies against site I of AD-2, we made libraries of antibody fragments based on one of the existing human antibodies reactive with this epitope, ITC88 (PAPER III). These libraries were produced by grafting of *in vivo* derived human CDR as well as synthetic CDR into the framework encoded by the V region genes utilized by this antibody. This technique has been termed CDR-shuffling (JIRHOLT et al., 1998), and has been used to create a large single-framework scFv library from which numerous specificities have been isolated, and also to modify selected scFv (SÖDERLIND et al., 2000; JIRHOLT et al., 2001; 2002; ELLMARK et al., 2002a; 2002b). The use of *in vivo* derived CDR sequences for the grafting is believed to ensure that the inserted loops are optimally functional as they have been proofread and selected for functionality during the formation of the B cell receptors.

The produced libraries were screened for binders to synthetic peptides that mimic site I of AD-2 (MEYER et al., 1992; OHLIN et al., 1993; 1996; SILVESTRI et al., 1993) by antibody phage display. This technique, which is based on the presentation of antibody fragments on the surface of bacteriophages and subsequent enrichment of phages that carry specific binders (WINTER et al., 1994; HOOGENBOOM et al., 1998), has been utilized for the study and isolation of antibodies against numerous targets, including viruses. For example, Burton *et al*. have isolated Fab fragments from immune phage-displayed libraries against a number of viruses including HIV-1 (BURTON et al., 1991; DITZEL et al., 1995), respiratory syncytial virus (BARBAS et al., 1992), herpes simplex virus 1 and 2 (BURIONI et al., 1994; SANNA et al., 1995), ebola virus (MARUYAMA et al., 1999), and measles virus (DE CARVALHO NICACIO et al., 2002). Similarly, others have isolated scFv from immune libraries against hepatitis C virus core and envelope peptides (CHAN et al., 1996) and from a synthetic library against rabies virus (RAY et al., 2001), and the technique has also been used to characterize antibody repertoires against vesicular stomatitis virus (LÓPEZ-MACÍAS et al., 1999). In addition, phage display has previously been utilized to dissect the potential light chain usage of the ITC88 antibody that served as a framework in this study (OHLIN et al., 1996).

When analyzing the scFv that were obtained after multiple rounds of selections on decreasing amounts of antigen and/or by competitive elution, it became evident that the selected repertoire displayed restrictions in terms of CDR sequence usage. Despite a library design that allowed for the incorporation of a highly diverse repertoire of CDR sequences (OHLIN et al., 1998), the selected clones all displayed some common features. In particular, it became apparent that CDRH1 and CDRL1 encoded by the germline genes that the parent antibody originates from (IGHV3-30 and IGKV3-11) were not compatible with effective interaction with the epitope. For instance, nearly all of the selected CDRH1 sequences encoded an aspartate at position 34. The IGHV3-30 germline gene encodes an alanine at this position, but the affinity matured ITC88 antibody also carries the aspartate. The only gene within the IGHV3 subgroup that encodes an aspartate at this position in its germline configuration is IGHV3-13, and the selected CDRH1 sequences were actually more similar to this gene than IGHV3-30. In order to further assess the importance of this mutation, we produced a variant of the parent scFv with this residue mutated back to an alanine. Evaluation of the binding of this variant to both a synthetic epitope-mimicking peptide and intact, recombinant gB showed that this residue was apparently critical for effective recognition of the epitope (PAPER III: Figs. 2 and 3). Position 34 in the heavy chain is frequently an antigen-contacting residue (MACCALLUM et al., 1996), and its importance in the interaction with the antigen has been demonstrated in other systems as well (KOBAYASHI et al., 1999; JIRHOLT et al., 2001). In a structure model of the parent scFv, this residue is located in close proximity to the apex of the CDRH3 loop (FIG. 3), suggesting that it, if not making direct contact with the antigen, may affect antigen recognition by influencing the conformation of other CDR loops. In this context, it might be worth noticing the somewhat poor recognition of the antigen-mimicking peptide by the clones based on the ITC88 scFv format with insertions after residues 32 and 33 in the heavy chain that were studied in PAPER II. This behavior may also be due to the proximity to the aspartate at position 34, as alterations in the vicinity of this residue may change the local conformation of the loop and thus make the interaction with the epitope less optimal.

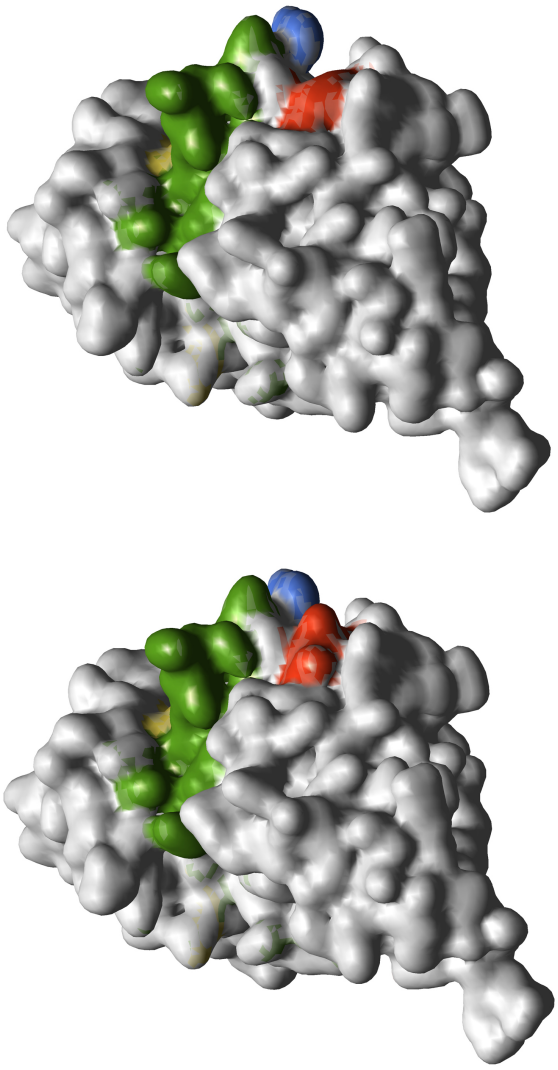

FIG 3. Structure models of the AE11F scFv that is based on the parent antibody ITC88 (top) and a variant scFv carrying the germline-encoded serines at positions 30 and 31 in the light chain (bottom). The models were generated using the WAM algorithm (WHITELEGG & REES, 2000), and the images were created using Weblab ViewerLite 3.2. CDRH3 is shown in green, whereas the aspartate at position 34 in CDRH1 and the tryptophan at position 113 in CDRL3 are shown in yellow and blue, respectively. The two residues at positions 30 and 31 in CDRL1, which are glycines in the topmost structure model, are highlighted in red.

The light chain CDR sequences that were selected displayed similar restrictions as the CDR of the heavy chain. For example, a highly conserved motif, including a tryptophan at position 113 in CDRL3, was found in nearly all

selected clones. This same motif was also retrieved from a light-chain shuffled library in a previous study (OHLIN et al., 1996), which indicates that it may be critical for the interaction with the epitope. Furthermore, although clones carrying the IGKV3-11 germline sequence in CDRL1 were also selected, a double mutation from serine-serine to glycine-glycine at positions 30 and 31 is required for effective interaction with the intact gB, as shown in PAPER V. The same double mutation is also found in the original antibody. The fact that the clones carrying the germline-encoded motif in CDRL1 actually dissociate somewhat slower from the peptide antigen than the parent scFv AE11F (PAPER III: Table 4), but dissociate more than ten times faster from intact gB (PAPER V: Table 1), may seem contradictory to the recognition of a common epitope by these scFv. However, as mentioned in chapter 2, it is well known that the buried surface area is larger in antibody-protein complexes than in antibody-peptide complexes. If one compares the structure models of the original AE11F scFv and a scFv that carries the germline-encode serines at positions 30 and 31 in CDRL1, the effect these mutations have on the antigen-binding site is evident (FIG. 3). While allowing for relatively effective binding to the peptide antigen, the additional bulges of the larger serine side chains most likely reduce the complementarity between the antigen-binding site and the larger contact surface on the protein antigen, thus making the interaction less optimal. Furthermore, the characterization of the fine-specificity of the different clones that was performed in PAPER V showed that the clones with the unmutated germline motif in CDRL1 actually displayed a slightly different fine-specificity than the parent scFv. This difference may naturally also affect the recognition of the epitope within the intact protein.

Due to the important role of gB in HCMV infection (NAVARRO et al., 1993; TUGIZOV et al., 1994; BOLD et al., 1996) and the potent neutralizing capacity of antibodies specific for site I of AD-2 (MASUHO et al., 1987; OHLIN et al., 1993), we decided to investigate what the factors are that influence neutralization via this epitope. The different mechanisms that are responsible for antibody-mediated virus neutralization in general have recently been reviewed by Klasse and Sattentau (KLASSE & SATTENTAU, 2001; 2002). They have described a number of potential steps in the viral replication cycle that antibodies may

interfere with, including virus attachment to target cells, post-attachment interaction with cellular receptors, internalization by endocytosis, fusion with the cell surface or endosomal vesicles, uncoating or appropriate intracellular localization of the core or capsid, and metabolic events catalyzed by viral enzymes, such as transcription. There is clear evidence of antibodies interfering with the first four steps, while the role of antibodies in the last two steps, i.e. post-internalization events, remains to be established (KLASSE & SATTENTAU, 2001; 2002). Thus, inhibition of virus attachment and interference with early entry events seem to be the dominant mechanisms of virus neutralization. Moreover, accumulating data indicate that antibody coating of virions is both necessary and sufficient for virus neutralization, which points to a blocking of interactions with target cell receptors or accessory molecules as the most important role of antibodies in virus neutralization (BURTON et al., 2000; 2001; ZINKERNAGEL et al., 2001; KLASSE & SATTENTAU, 2002). According to the occupancy model by Burton *et al*., virus neutralization occurs when antibodies occupy a sufficient number of binding sites on the virion, thereby blocking key interactions with the target cell and making the virus unable to infect (BURTON et al., 2000; 2001). Antibodies specific for site I of AD-2 have been shown to exert their virus-neutralizing effects without preventing primary binding of the virions to the target cells (OHIZUMI et al., 1992; GICKLHORN et al., 2002), but the exact role of the antibodies in this process is unknown. Interestingly, this epitope on gB has been shown to bind to both heparin and the extracellular matrix of human cells, indicating that it may function as a ligand to heparan sulphate proteoglycans (SILVESTRI & SUNDQVIST, 2001). However, the fact that virions can bind to cells even in the presence of ITC88 (GICKLHORN et al., 2002), suggests that this site is not the only ligand for cellular heparan sulphate on the virions.

In PAPER IV, we analyzed the HCMV neutralizing capacity of the scFv format of the neutralizing ITC88 antibody (AE11F) in its monomeric and dimeric forms in order to elucidate the effects of antibody valency on virus neutralization via site I of AD-2. We found that the monomeric scFv did not neutralize infection, as determined by a plaque assay, whereas the spontaneously formed dimeric scFv effectively neutralized the virus. However, upon

dimerization of the monomeric scFv using a secondary antibody against a C-terminal tag on the scFv molecules, neutralizing capacity was reestablished, at a level comparable to the spontaneously dimerized scFv. Similar effects on the neutralizing capacity of antibody fragments have been seen with other enveloped viruses, such as HIV-1 (CAVACINI et al., 1994), murine hepatitis virus (LAMARRE & TALBOT, 1995), and varicella-zoster virus (DREW et al., 2001), indicating that this behavior is not unique for HCMV. In order to determine whether the restored neutralization was an effect of higher functional affinity, or avidity, of the divalent formats, we analyzed the reaction rate kinetics of monomeric and dimeric scFv with both streptavidin-bound biotinylated epitope-mimicking peptide and intact, recombinant gB. Although the dimeric scFv had a much lower dissociation rate with the peptide antigen than the monomers, both formats displayed similar dissociation rates with intact gB (PAPER IV: Fig. 3). Thus, avidity did not seem to determine the neutralizing capacity of the AE11F scFv. In this context, it may be argued that the peptide is much more efficiently displayed as each streptavidin molecule has four binding sites for biotin, while the recombinant gB may not present as many epitopes in close proximity to each other for avidity to influence the dissociation. However, an analysis of the binding of monomeric and dimeric scFv to HCMV infected human cells, which should contain natural gB dimers (BRITT & VUGLER, 1989; 1992), confirmed that both formats bound to the same extent (PAPER V).

Thus, it seems as though a bulkier molecule than the monomeric scFv is required to block the post-attachment events that lead to HCMV infection. The fact that an intact antibody molecule is approximately the same size as the envelope spikes of for instance HIV-1, has been offered as further evidence for steric hindrance of virus-cell interactions as the most important mechanism for antibody-mediated virus neutralization (BURTON et al., 2000; 2001). The size of dimeric gB complexes on the surface of HCMV virions is approximately 340 kDa (BRITT & VUGLER, 1992), which is roughly the size of two IgG molecules (150 kDa). The complexes formed by dimerization of monomeric scFv (27 kDa) using a secondary (IgG) antibody are thus clearly bulky enough to provide the steric hindrance that is required according to this line of reasoning. The dimeric scFv are, however, only one third the size of one gB molecule, and it is rather

unclear whether this is enough to block the secondary interaction that leads to infection by steric hindrance only. It has been suggested that antibodies may induce conformational changes in the target molecules on the virions, thereby rendering them non-infective (see for instance (HEWAT & BLAAS, 2001; ZINKERNAGEL et al., 2001). Furthermore, crosslinking of epitopes by divalent antibodies has been suggested to inhibit conformational changes in gp120 of HIV-1 that are required for the infective process (CAVACINI et al., 1994). However, as the selection mechanism that decides which antibody-producing B cell clones are expanded in response to antigenic challenge is believed to be based on affinity for the antigen, not the ability to perform some secondary functions (RAJEWSKY, 1996), this has been suggested to be an unlikely mechanism for virus neutralization (BURTON et al., 2001; KLASSE & SATTENTAU, 2001). Further studies will be required to conclusively determine whether it is the size or the valency of the dimeric antibody fragments that is responsible for the neutralization of HCMV via site I of AD-2.

In PAPER V, we characterized a number of the clones that had been selected by phage display in PAPER III in terms of binding to gB, both as recombinant protein and in its natural form in HCMV-infected cells, fine-specificity of the interaction with the epitope, and virus neutralizing capacity. Due to the restricted CDR sequence usage of these clones, they may be regarded as a repertoire of antibodies that has arisen by somatic diversification of a single clone. The results from this study strongly indicated a correlation between the binding of the individual scFv clones to recombinant gB and their virus neutralizing capacity. We found that all of the clones that were able to neutralize the virus had half-lives longer than one hour on the intact gB, whereas the non-neutralizing clones had half-lives of approximately ten minutes. This poor binding to the recombinant antigen was also reflected in the binding to HCMV infected cells, as the non-neutralizing scFv only gave rise to very weak staining in the immunofluorescence assay (PAPER V: Table 1). These results further indicate that efficient coating of the virion, as suggested by Burton *et al*. (BURTON et al., 2000; 2001), rather than induction of conformational changes in gB, is the mechanism responsible for the neutralization of HCMV by antibodies specific for site I of AD-2. As the antibody-virion complexes were incubated

with the target cells for one hour in this study, it is likely that the rapid dissociation of the non-neutralizing clones exposed enough sites on the virions to allow for infection to proceed. Furthermore, the findings also reinforce the observation from PAPER III that mutations at key positions in the germline sequence are required for effective interaction with the antigen, since the three non-neutralizing antibodies all carry the unmutated serine-serine motif or a highly related motif in CDRL1.

When analyzing the fine-specificity of this repertoire of scFv specific for site I of AD-2, it became clear that they displayed a range of reactivity patterns. It has been observed previously that both chain-shuffled scFv variants and antibodies isolated from immune sera can recognize this epitope in multifarious ways (SILVESTRI et al., 1993; OHLIN et al., 1996), and it has been found that only serum antibodies having the same fine-specificity as the ITC88 antibody are able to reduce HCMV infection (SILVESTRI et al., 1993). Importantly, we found in PAPER V that the non-neutralizing clones displayed a fine-specificity resembling that displayed by similarly non-neutralizing antibodies purified from immune sera from both humans and rabbits (SILVESTRI et al., 1993). It seems thus as this neutralization epitope can give rise to an array of antibodies, exhibiting different fine-specificities and neutralizing capacities, *in vivo* as well as by the *in vitro* evolution techniques used here and previously. The fact that some of the antibodies that are specific for site I of AD-2 are non-neutralizing raises the question whether they may compete with the neutralizing repertoire for the binding sites on the virions and thus reduce the protective potential of this. The existence of binding, but non-neutralizing, antibodies has been argued against by Burton and colleagues (BURTON et al., 2001), but as they admit themselves, such antibodies may simply be absent in the systems they have studied. In the HCMV system, competitive binding of various antibodies against the immunodominant epitope on gB, AD-1, that leads to reduced or completely abolished virus neutralization, has in fact been reported (UTZ et al., 1989; SPECKNER et al., 1999). It has also been suggested that it is an evasive strategy of the virus to induce a multitude of antibodies, which, when combined, only gives partial protection from infection (SPECKNER et al., 1999). The non-neutralizing clones found in this study are admittedly of low affinity, and further

characterization is required in order to determine the actual impact they may have on the neutralizing potential of other clones.

The results from PAPERS III-V may be put to use for the design of an improved subunit vaccine according to what has been termed reverse vaccinology (BURTON, 2002). The primary concept of this technique is to generate vaccines from the knowledge of the interactions between antibodies and envelope proteins, such as gB. In line with this idea, it may be suggested that an improved immune response against site I of AD-2 could possibly be obtained *in vivo* if immunogens, in which the normally immunodominant AD-1 is made less immunogenic, were employed in vaccine strategies. This approach could potentially eliminate the effects of competing responses, but the effectiveness of such a strategy remains to be evaluated. The fact that the antigenicity of a protein can be altered significantly by mutating a single critical residue in the epitope recognized by monoclonal antibodies without destroying the biological function of the protein has been known for some time (ALEXANDER et al., 1992). Recently, it was also shown that by mutating a single residue in recombinant human chorionic gonadotrophin, the immune response was refocused to another, normally weakly immunogenic region (CHIESA et al., 2001). Similarly, mutation of a single residue in the V3-loop of HIV-1 gp120 has been demonstrated to shift its immunity away from a humoral response altogether (LEE et al., 2000). Taken together, these data suggest that it may be possible to develop a recombinant form of gB that, while carrying all the important features of AD-2, would not evoke a strong response towards AD-1.

This approach may enhance the possibility to recruit B cell clones that are reactive with AD-2 at a higher frequency than in the presence of the strongly immunogenic AD-1. As indicated by the low affinity of the clones carrying the germline-encoded motifs (PAPERS III and V), the initially selected germline-encoded clones would subsequently have to affinity mature in order to effectively recognize the antigen. In the absence of competition for the antigen from other specificities, this may take place at a higher frequency than during natural HCMV infection, and thus shift the gB-specific repertoire towards AD-2. However, the principle of original antigenic sin must be taken into consideration, as it may prevent the development of clones that recognize the

natural viral antigen. According to this principle, neutralizing antibodies initially directed against a single virus strain may interfere with the ability of the immune system to mount an adequate response against a second challenge by an antigenically different virus strain (FAZEKAS DE ST.GROTH & WEBSTER, 1966a; 1966b). Experiments with defined protein antigens (myoglobins) that are related to each other, but differ in amino acid sequence in the antigenic regions, have further confirmed that this phenomenon may affect the secondary antibody response (EAST et al., 1980). B cell clones that have been recruited into the repertoire by immunization with the recombinant form of gB may thus influence the response against the natural immunogen upon HCMV infection. However, as the main objective of this approach is to evoke a stronger response against AD-2 by presenting the consensus viral epitope in a more favorable manner, the response against AD-2 as displayed on the virus does not necessarily have to be impaired. In addition, since the AD-1 region that is presented on the recombinant antigen is supposed to lack immunogenicity, there should not be a strong response against the mutated version of this epitope that could subsequently interfere with the development of a response against the native viral epitope. Speculations aside, only experimentation can determine whether strong, protective antibody responses will develop against these epitopes by the proposed vaccination strategy.

6 CONCLUDING REMARKS

In PAPER I, we demonstrated that CDR1 and CDR2 of human IGHV germline genes specifically carry accumulations of trinucleotide repeats. This feature of repetitive sequence tracts very likely targets these regions with insertion and deletions during the somatic hypermutation process, as is also evidenced by the natural occurrence of single-codon deletions in regions of highly repetitive sequence character. The fact that these accumulations are found in the CDR indicates that the germline gene repertoire has evolved in a way that ensures that modifications of antibody sequence and structure are targeted to the regions of the molecules that, due to their flexible nature, can be expected to tolerate them. Furthermore, since genes belonging to different IGHV subgroups have their foci of repeats at different positions in the CDR, it may be possible that the subgroups have evolved in response to different types of antigens, and are therefore unequally suited to participate in the response against these antigens. Another finding from this study, which may explain why antibody light chain V genes are less frequently targeted by insertion and deletion events (GOOSSENS et al., 1998; DE WILDT et al., 1999b), is that similar trinucleotide accumulations are not present in the IGKV and IGLV germline gene repertoires, with the exception of a few genes.

As the number of human antibodies with known specificities that carry insertions or deletions in their V regions is very limited, little is known about the consequences of these sequence modifications. However, in PAPER II we were able to show by antibody engineering that single-codon insertions and deletions are well tolerated in CDRH1 and CDRH2 of antibody fragments belonging to the IGHV3 subgroup, as antigen recognition was frequently retained and the stability of the modified proteins seemed unaltered. As the modifications created new canonical loop structures and combinations of loop structures not found within the IGHV3 subgroup, or even within the human germline repertoire, this finding indicates that the repertoire of CDR loop structures may not be as restricted as previously thought. The results also demonstrate the plasticity of IGHV3-derived V region frameworks, and indicate further that this approach of modifying antibody structure may be an useful way of improving existing

specificities or create new ones. Insertions in the CDRH2 loops of antibodies have admittedly been used previously to improve the binding of hapten-specific murine antibodies (LAMMINMÄKI et al., 1999; PARHAMI-SEREN et al., 2002), but it may be envisaged that this mode of modifying the structure of the CDR loops can be used in a more general way to create antigen-binding sites with topographies that are highly complementary to a specific class of antigens (VARGAS-MADRAZO et al., 1995; LARA-OCHOA et al., 1996). This approach can be used to create libraries of antibodies with, for instance, a cleft-like binding-site for the selection of binders that are specific for small molecules, such as haptens. It may also be combined with the introduction of residues that are known to be of importance for the interaction with certain antigens, such as aromatic and hydrophobic residues in the case of many haptens (SAWADA et al., 1991; AREVALO et al., 1993a; 1993b; LAMMINMÄKI & KANKARE, 2001). Others have used a similar approach in an effort to create antigen-binding sites that would be complementary to epitopes on proteins in terms of both topography and distribution of charged residues (SCHIWECK & SKERRA, 1997; KIRKHAM et al., 1999). Furthermore, since entirely new regions of antibody sequence and structure space may be explored by this approach of codon insertion and/or deletion, it may even be possible to target poorly immunogenic or previously unrecognized epitopes. In conclusion, the results from PAPERS I and II indicate that the process of insertion and deletion of residues in the CDR is a highly useful way of increasing antibody sequence and structure space *in vivo* as well as *in vitro*.

In PAPER III, we studied the development of antibodies against a poorly immunogenic epitope on glycoprotein B of human cytomegalovirus, site I of AD-2, by using phage display to select binders from CDR-shuffled libraries of antibody fragments. The results from this study showed that although the V genes that the parent antibody originates from have certain features that make them superior to other, related genes in the creation of a paratope specific for this antigenic determinant, they are still not optimally suited for the recognition of the epitope. We found that mutations at key positions in both CDRH1 and CDRL1 were required for effective recognition of the epitope, especially as it is presented in the intact viral glycoprotein. Although the occurrence of these

mutations is not entirely unlikely *in vivo*, as demonstrated by their relatively frequent encounter in hypermutated human antibodies, the requirement for them seems to be a clear disadvantage for the development of an antibody response towards site I of AD-2 *in vivo*. Other epitopes, such as the immunodominant AD-1, for which there may exist an imprinted specificity in the human IGHV germline gene repertoire (OHLIN et al., 1994), very likely have an advantage in the competition for the available antigen during the onset of the immune response, and will therefore become the dominant specificities of the antibody repertoire against gB. The fact that antibodies specific for site I of AD-2 seem to require mutations in order to achieve high-affinity binding is actually reflected in the kinetics of antibody formation following HCMV infection. Antibodies against this epitope are practically absent upon primary infection and are produced significantly slower than AD-1 specific antibodies during virus reinfection or reactivation (SCHOPPEL et al., 1997). Thus, based on the only known sequence of a human antibody specific for site I of AD-2, we conclude that the human antibody germline-encoded repertoire apparently does not have members that are ideally suited to recognize this epitope at high frequency. Rather, certain mutations that target the CDRH1 and CDRL1 of low-affinity, yet specific, clones are required to produce a high affinity response. Although this study does not rule out other solutions to the generation of a paratope specific for site I of AD-2, the results indicate that the poor immunogenicity of this epitope is a consequence of the lack of an imprinted specificity in the germline-encoded repertoire that effectively recognizes the epitope. These findings may provide some insights into the development of antibodies to exogenous antigens in general, and against a weakly immunogenic, but potently neutralizing epitope on human cytomegalovirus in particular.

In the two remaining papers, PAPERS IV and V, we investigated the basis for the virus neutralization by antibodies specific for site I of AD-2, and also studied the epitope recognition and virus neutralizing capacity of a clonally related repertoire of antibody fragments specific for this epitope. The results indicate that a divalent antibody format is required for neutralization via this epitope, since monomeric antibody fragments are unable to block infection *in vitro*, whereas dimeric constructs with a molecular weight of less than 60 kDa are able

to mediate effective virus neutralization. Although avidity effects did not seem to make a difference in the neutralizing capacity of the different antibody formats, the neutralizing potential of the repertoire of antibodies selected in PAPER III was demonstrated in PAPER V to be determined by the reaction rate of the interaction with gB. All the non-neutralizing clones dissociated rapidly from the antigen, and, furthermore, displayed a fine-specificity of the interaction with the epitope that was significantly different from that of the neutralizing clones. Whereas these data agree with a neutralization mechanism based on occupancy of binding sites on the virion (BURTON et al., 2000; 2001), the exact mechanism of neutralization via site I of AD-2 still remains to be elucidated. The fact that gB is present as homodimers in the membrane of infectious virions (BRITT & VUGLER, 1992) may indicate that crosslinking of epitopes is required for effective neutralization to occur. A possible approach to determine whether this is the case, or if the steric hindrance provided by the larger size of the dimeric format alone can block the infection, is to expand the study with monovalent constructs of the same size as the spontaneously dimerized scFv, e.g. Fab fragments.

Based on the findings in PAPERS III-V, it would be highly interesting to investigate whether it may be possible to improve the response against this epitope by a reverse vaccinology strategy (BURTON, 2002), whereby the response against the immunodominant epitope AD-1 would be diminished so as to avoid competing responses. However, the finding in PAPER V that a repertoire of closely related antibodies specific for site I of AD-2 consists of both neutralizing and non-neutralizing clones, must be taken into consideration. Further studies, such as competitive binding experiments with virus neutralization as read-out, are required to define the influence of the weakly binding and non-neutralizing clones on the protective potential of a repertoire of antibodies specific for this epitope.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Antikroppar är viktiga beståndsdelar av det naturliga immunförsvaret som skyddar oss mot smittämnen, såsom bakterier och virus. Utvecklingen av olika genetiska och biokemiska teknologier har dessutom möjliggjort användningen av antikroppar för en mängd ändmål, bl a för diagnostik och behandling av infektionssjukdomar och cancer. I denna avhandling, som bygger på fem vetenskapliga originalartiklar, har jag undersökt olika aspekter av hur antikroppar kan utvecklas, både naturligt i den mänskliga kroppen och i laboratoriet, samt hur en repertoar av antikroppar kan uppstå som en följd av en virusinfektion, och hur denna repertoar kan skydda mot viruset.

Resultaten av mina studier visar att de mänskliga gener som kodar för antikroppar uppvisar vissa mönster i den genetiska koden som leder till att antikropparna kan genomgå betydligt större förändringar under utvecklingen än vad som tidigare ansågs ske. Dessa förändringar innebär att de delar av antikropparna som binder till främmande substanser kan variera ifråga om längd. Genom att i laboratoriet skapa funktionella antikroppar med sådana förändringar har jag visat att det är möjligt att utnyttja denna upptäkt för bioteknologiska ändamål. På detta vis kan man få fram antikroppar som uppvisar förbättrade egenskaper ifråga om igenkänningen av vissa mål, men tillvägagångssättet kan möjligen även användas för att skapa antikroppar mot mål som sällan ger upphov till antikroppar på naturlig väg.

En annan upptäckt som min forskning har lett fram till är hur antikroppar kan uppstå som svar på en virusinfektion. Viruset det rör sig om är cytomegalovirus, ett herpesrelaterat virus som orsakar problem för individer med nedsatt immunförsvar, t ex nyfödda. Detta virus ger upphov till en mängd antikroppar som är riktade mot ett antal strukturer på dess yta. En viss sådan struktur kan ge upphov till antikroppar med starkt skyddande effekt mot cytomegalovirusinfektion, men detta sker inte i de flesta individer som bär på viruset. Jag har upptäckt att detta antagligen beror på att den uppsättning antikroppsgener vi bär på inte är anpassad för att skapa antikroppar mot denna struktur, utan det krävs sällan förekommande förändringar av antikropparna för att de ska erhålla den starkt skyddande effekten. Denna upptäckt kan förhoppningsvis, tillsammans med de upptäckter jag gjort gällande hur antikropparna skyddar mot viruset, användas för utveckling av ett effektivare vaccin mot viruset än de i nuläget tillgängliga vaccinerna.

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