

Chapter 4.2

HUMAN MONOCLONAL ANTIBODIES FOR PROPHYLAXIS AND THERAPY OF VIRAL INFECTIONS

J. TER MEULEN and J. GOUDSMIT

Crucell Holland B.V., Archimedesweg 4, 2301 CA Leiden, The Netherlands

Abstract: Monoclonal antibody (mAb) technology has reached a state of maturity making it possible to rapidly discover and produce fully human mAbs which neutralize any given virus *in vitro*. Several antibodies have also shown good prophylactic and therapeutic efficacy in relevant animal models of viral disease. Surprisingly, only one antiviral mAb has been licensed to date; it is used to prevent infection with respiratory syncytial virus in at-risk infants. Certain paradigms of mAbs prevailing in industry and academia are obstacles to the development of further antiviral antibodies for clinical use. There is a perception that mAbs are much more effective in prophylactic than therapeutic use, offer limited breadth of protection against different viral strains, are prone to select neutralization escape variants and need to be given in high doses, which are still comparatively costly to manufacture. Based on a review of the literature and our own data, we propose that by combining two or more non-competing neutralizing antibodies, viral escape can effectively be controlled and in case of synergistic action of the mAbs it may be possible to significantly reduce the total antibody concentration required for protection. The combination of mAbs with specific antiviral drugs has the potential to expand their use from pre- and post-exposure prophylaxis to therapy of acute and chronic viral infections.

1. INTRODUCTION

Before the advent of specific vaccines and antiviral drugs, passive prophylaxis with pooled normal donor serum (“immune globulin”) or pooled pathogen-specific (“immune”) serum from recovered patients or immunized volunteers was used very successfully to prevent infections with a variety of

viruses, such as cytomegalovirus, enteroviruses, hepatitis A, hepatitis B, measles, parvovirus B19, rabies, respiratory syncytial virus, varicella and variola (reviewed by Casadevall *et al.* 2004 and Sawyer 2000). Despite high production costs and the inherent risks of transmitting known and unknown blood borne pathogens, many of these sera are still in clinical use. Important indications are the protection of individuals at high risk of infection or at high risk of developing complications after infection. The immune globulin or pathogen-specific immune serum is given either as prophylaxis before or immediately after exposure (post-exposure prophylaxis, PEP) to immunocompromised or normal persons. It is used less frequently for therapy of acute or chronic infections in immunocompromised patients.

Typical prophylactic indications include RSV-specific antibodies for at-risk infants, hepatitis B or CMV immune serum for transplant patients, varicella zoster and measles virus antiserum for exposed immunocompromised patients and immunoglobulins for patients with X-linked agammaglobulinemia. However, in the case of highly lethal diseases, immunocompetent persons also benefit from passive immunization. Rabies virus kills 30,000 to 40,000 people annually in Asia and the shortage of affordable high quality rabies immunoglobulin for PEP given in combination with the vaccine is considered a serious global health problem.

CMV-immunoglobulin plus ganciclovir is the treatment of choice for established pneumonia in bone marrow and stem cell transplant patients. Other acute viral infections showing some, mostly only anecdotally documented response to immunotherapy include the complications of smallpox vaccination (eccema vaccinatum, generalized vaccinia), Lassa fever, West Nile virus infection, SARS and Ebola virus infection.

Of the chronic viral infections, enteroviral encephalitis and parvovirus B19 anemia in immunosuppressed patients are amenable to immunoglobulin therapy. In contrast, HIV and hepatitis B and C, which affect hundreds of millions of people worldwide, have shown little responsiveness to serum therapy. Possible reasons include low-titered or virus strain-specific neutralizing antibodies in donor sera, generation of neutralization escape variants and requirement of a strong T-cell response for clearance of infected cells.

From the above it can be concluded and has been shown experimentally in animal models, that for pre- and post-exposure prophylaxis of viral infections human immune sera can effectively be replaced by monoclonal antibodies (mAb), provided the mAb offers sufficient breadth of protection against all clinically relevant strains of a virus. It also follows that late PEP or early therapy of an acute infection will be possible in certain circumstances, provided that the main mechanism of viral clearance is antibody mediated and that the mAb reaches sufficient concentrations in the

target organs of viral replication (e.g. the brain). Chronic infections, however, are very unlikely to respond to therapy with mAbs unless immune escape is prevented by reducing viral replication independently of antibody action (e.g. by an antiviral drug) and the immune response is modulated such that additional effector mechanisms for viral clearance are stimulated.

Murine mAbs against transplant rejection and blood clotting were the first monoclonal antibodies to enter the clinic in the late 1980s, but the induction of immunological side effects was an obstacle to the development of further products. The advent of recombinant antibody technology in the 1990s then allowed the generation of less immunogenic chimeric mouse-human and later “humanized” mouse mAbs, which has set off an annual 20% growth of the mAb market to currently 30 billion US\$/a (Baker 2005). At present 152 novel mAbs are undergoing clinical trials, mostly for cancer or immunological disorders and approximately 10% for infectious diseases. Despite these successes, only a handful of antiviral antibodies are currently in clinical trials, mainly against HBV and HCV. Only one mAb is licensed and marketed for prevention of a viral disease (Palivizumab), which is given to at-risk infants for the prevention of infection with respiratory syncytial virus. While economic considerations with respect to the smaller market size of anti-infectives and comparatively high production costs of mAbs may partly explain this discrepancy, we think that certain scientific paradigms prevailing in industry and academia are important obstacles to the development of further antiviral antibodies for clinical use. The following chapter will therefore try to shed light on the generation and preclinical evaluation of antiviral mAbs and discuss possibilities to improve their properties based on our own data and a review of the literature.

2. GENERATION OF HUMAN MONOCLONAL ANTIBODIES AND EVALUATION OF THEIR ANTIVIRAL PROPERTIES

2.1 Technical approaches

Immunoglobulins are composed of heavy and light chains, the light chain being either κ or λ . The antigen-binding site of an antibody is composed of six hypervariable or complementarity determining regions (CDRs), of which three are located within the variable domains of the light-chain (V_L) and heavy chain (V_H), respectively. In the immune system a large repertoire of different variable domains determining the antibody specificity is generated through combinatorial assembly of germline gene segments (V, D and J),

with each B-cell expressing a different antibody specificity. Antigen exposure leads to clonal expansion of those B-cells producing antigen-binding antibodies and induces somatic mutations in the V genes, which results in further selection of clones with improved affinities of the antibodies (affinity maturation).

Of the 18 therapeutic mAbs in clinical use today, three are murine, five are chimeric (murine V region, human F_C region) and the remainder are humanized (mouse CDRs grafted onto a human antibody). Because fully human mAbs will theoretically not induce any immune response in the patient, several *in vivo* and *in vitro* technologies were developed for their generation (reviewed by Hoogenboom 2005). Historically, *in vitro* immortalization of peripheral human B-cells isolated from infected or immunized donors was performed with Epstein Barr virus to subsequently isolate and identify antibody secreting clones (Steinitz *et al.* 1977). This technique has recently been modified to increase the number of transformed IgG⁺ B-memory cells by *in vitro* stimulation with CpG-oligonucleotides and sorting of memory cells using the surface marker CD22, which has resulted in recovery of a high number of specific antibody clones, which have undergone in-vivo affinity maturation (Traggiai *et al.* 2004). Transgenic mice with human immunoglobulin genes have been generated to study the maturation of antibody genes. They are immunized with the antigen of interest and monoclonal antibodies are rescued by fusing mouse B-cells with mouse myeloma cells (Lonberg 2005). The resulting antibodies are in-vivo affinity-matured.

Ribosome display of antibodies is a PCR-based *in vitro* display technology, in which the individual nascent proteins are coupled to their corresponding mRNA through formation of stable protein-ribosome-mRNA complexes. Thus it is possible to isolate the functional protein (antibody) together with its encoding mRNA, which is reverse transcribed and PCR-amplified for further rounds of selection (Lipovsek *et al.* 2004). Due to the error-prone process of reverse transcription and amplification, the system's most successful application is the affinity maturation of antibodies.

Surface display on yeast (*S. cerevisiae*) allows for selection of antibody repertoires by cell flow cytometry. The V_H and V_L genes are diversified with random mutagenesis to yield high affinity antibodies (Boder *et al.* 2000). Other formats including surface display on *E. coli* and *Bacillus* strains, retroviral display, display based on protein-DNA linkage and others have either specific technical disadvantages or are not yet mature enough for commercial use.

Phage display is currently the most popular molecular technology used to tap into the human antibody repertoire (Hoogenboom 2005). As table 1 shows, *in vitro* and *in vivo* neutralizing human monoclonal antibodies have been generated against a variety of virus from diverse families by phage display.

All variable, antigen binding regions of the heavy and light chains, termed V_H and V_L , are cloned from the lymphocytes of a donor and expressed as single-chain antibodies (V_H - V_L) fused to the pIII surface protein of a bacteriophage. The resulting vast repertoire (“library”) of different antibody carrying phages ideally mirrors the antibody repertoire of the donor. Cloning of all different antigen binding regions is possible because the hypervariable regions which are responsible for antigen specificity (CDRs) are flanked by constant regions (framework regions, FR) which correspond to families of germline antibody genes for which the genetic sequences are available. Because the heavy and light chains are amplified and cloned by two separate PCR reactions, the library also contains pairings not present in the donor lymphocytes, which further increases its size. The antibody phages are incubated with the antigen of interest and specifically binding phages are rescued together with the genetic information coding for the specific antibody gene. Both the cloning and the selection procedure are very powerful, allowing essentially to generate a repertoire of $10^8 - 10^9$ different molecules and extracting the desired antibody specificity from a single test tube.

For further characterization, antibody genes are recovered from the phages, recloned in appropriate vectors and transiently expressed in eukaryotic cell lines as fully functional monoclonal antibodies of a desired class and subclass. Starting from high-quality donor material, a typical antiviral mAb discovery program may yield 1000-2000 virus binding antibody phages, of which 100-200 are converted to IgG1-molecules for further *in vitro* and *in vivo* screening. Finally, three to four antibodies are selected as product leads and stable production clones of a suitable cell line (e.g. PER.C6[®]) are generated (figure 1). Excellent laboratory manuals are available describing the technical procedures in detail (Kontermann and Dübel 2001).

Table 1. In-vitro neutralizing human monoclonal antibodies generated by phage display or other methods

Virus	Target	In-vivo neutral.	Reference
CMV	gB, gH	nd	Nejatollahi 2002
Dengue1,2	E	nd	Goncalvez 2004
Ebola	GP	Guineapig	Parren 2002
Hantaan	G1,G2	nd	Koch 2003, Liang 2003
HAV	14S, 70S, subviral particles	nd	Kim 2004
HBV	Pre-S, S	Chimpanzee	Hong 2004 ^c , Eren 2000 ^b
HCV	E1, E2	nd	Keck 2004 ^a , Habersetzer 1998 ^a
HEV	ORF 2	Rhesus	Schofield 2003
HIV-1	gp120 (V2, V3), gp41 CD4/co-receptor complex	Chimpanzee, rhesus SCID- mice	He 2002 ^b , Moulard 2002, Wang 1999, Purtscher 1994 ^a
HIV-2	gp120	nd	Bjorling 1999
Measles	H	nd	De Carvalho Nicacio 2002
Parvo	VP2	nd	Arakelov 1993 ^a
Puumala	G2	nd	De Carvalho Nicacio 2000
Rabies	G	Hamsters	Bakker 2005, Hanlon 2001 ^a , Ennsle 1991 ^a
Rotavirus	VP4, VP7	nd	Higo-Moriguchi 2004
RSV	F	nd	Johnson 1997 ^c
SARS	S1, S2	Mice, ferrets	Greenough 2005 ^b , ter Meulen 2004, Sui 2004, Traggiai 2004 ^a
Vaccinia	p95, p34	nd	Schmaljohn 1999
VZV	gE	nd	Kausmally 2004
Yellow fever	E	nd	Daffis 2005

a Immortalized human B-cells b Transgenic mice c Humanized murine mAb

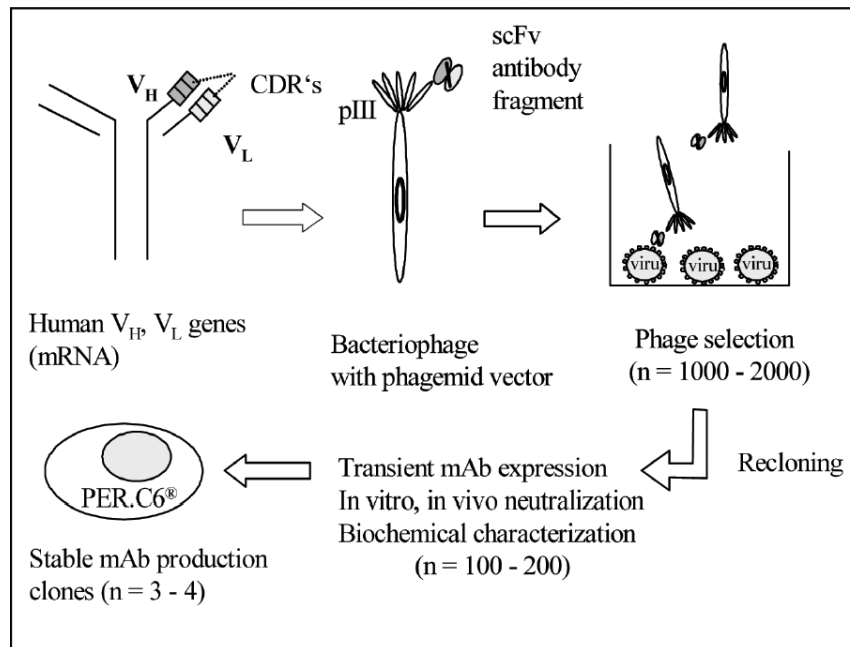


Figure 1. Generation of human monoclonal IgG antibodies from phage display libraries. mRNA is isolated from immunized or infected lymphocytes donors, reverse transcribed into cDNA and all V_H and V_L genes are amplified using a set of approx. 40 consensus primers directed against the conserved framework regions of the variable antibody genes. The antibody genes are cloned in a phagemid vector and infectious phages each expressing a different single-chain antibody molecule (the V_H and V_L chains being linked by an artificial polypeptide spacer) are rescued from bacteria using a defective helper phage (Kramer *et al.* 2003). The resulting “antibody phage library” is screened with viral antigen in different formats for specific binders during 2-3 rounds of selection. The rescued antibody genes are then recloned in appropriate vectors and transiently expressed as fully human IgG1 molecules. These are used to determine the potency, affinity and epitopes of the antibodies. A stably transfected production cell line is generated for each lead antibody. The whole process can be performed in approx. 9 months.

2.2. Libraries and antigens

The quality of the library and the quality of the antigens used for screening are the two major critical factors for a successful discovery of highly potent antibodies. Libraries generated from non-immune donors have been enlarged by introducing mutations in the CDRs of the V_H and V_L chains resulting in a size of up to 10^{10} different specificities, and are readily available for screening (de Kruif *et al.* 1995). Interestingly, these libraries

can yield potent neutralizing mAbs reactive with viruses against which the donors have no measurable serum antibody titer, such as SARS coronavirus (van den Brink *et al.* 2005).

Libraries from immunized or infected donors are smaller in size (typically 10^8), but with good timing concerning the sampling of the lymphocytes in relation to the time point of infection they contain a large number of affinity matured and therefore highly potent antibody specificities (Kramer *et al.* 2005).

Neutralizing viral epitopes are located on the glycoproteins in case of enveloped viruses or on the capsid proteins in case of non-enveloped viruses. Often these epitopes are discontinuous and sensitive to denaturation, so that they may not be preserved if purified virions are chemically or physically inactivated for safety reasons before selection. Similarly, recombinant viral envelope proteins may not adopt the native conformation under the expression and purification conditions used. Furthermore, surface proteins of viruses tend to adopt oligomeric configurations in which aminoacids from two molecules may contribute to one epitope (Daffis *et al.* 2005). It is therefore very useful to employ different antigen formats for screening of the libraries, including whole purified virions, virus like particles, recombinant proteins and eukaryotic cells expressing viral surface proteins. By performing the second round of selections on a different antigen format than the first, unspecific reactivity can be reduced. Certain combinations of antigen formats, e.g. selection on cells expressing viral surface proteins followed by selection on whole virions, may yield the majority of neutralizing antibodies recovered from a library (Marissen *et al.* 2005).

2.3. In vitro and in vivo characterization of antiviral mAbs

Potent neutralizing antibodies need to be selected as quickly and efficiently as possible from the large number of antigen binding phage antibodies, which can be in the order of many thousand. In principle, this is possible by characterizing the molecules biochemically by measuring e.g. their affinities to the virus, or functionally by determining their neutralizing potency. While the term “neutralization” in its broadest sense denotes the inhibitory effect of an antibody on viral infectivity or replication, it is more often specifically used when referring to the inhibition of viral attachment and early entry functions (endocytosis, fusion, uncoating). There has been some debate as to whether neutralization of viruses is due to binding of an antibody to one critical site on the virion (leading to a single-hit kinetic of neutralization) or to epitopes present in multiple copies on the virus surface

(multiple-hit kinetic). In the latter case neutralization can be regarded as a function of antibody affinity and concentration, which determine whether a critical number of antigens have reacted with the antibody (so called relative occupancy model of neutralization). Many experimental data point to the multiple-hit kinetic and occupancy model, with 4-7 IgG molecules required to neutralize picornaviruses, 70 to neutralize influenza and 225 to neutralize rabies virus. Interestingly, for several viruses a linear relationship between the number of antibodies required for neutralization and the particle surface area was found, supporting the idea that coating of the virion surface by any antibody is sufficient for neutralization (high occupancy or coating theory). Exhaustive reviews of the subject have been published by Parren & Burton 2001 and Klasse & Sattentau 2002. However, other molecular mechanisms of neutralization have also been proposed which may or may not follow the occupancy model, including induction of conformational changes in the capsid or envelope proteins, aggregation of viral particles, complement fixation and signalling to the interior of the virion or the infected cell.

Neutralization of HIV has been studied in great detail and revealed some unique features of the rare mAbs which are broadly cross-clade neutralizing (reviewed by Ferrantelli and Ruprecht 2002). Long, finger-like CDR3 loops have been found inserting into the narrow CD4 binding pocket of gp120 or displaying a hydrophobic patch which reacts with the membrane-proximal region of gp41 and the viral membrane (mAbs b12 and 2F5/4E10, respectively. Zwick *et al.* 2003, Zwick *et al.* 2005). The latter mAbs, which were recovered by phage display from HIV infected patients, were found to be autoreactive with cardiolipin, which possibly explains that their specificities are rarely detected in humans (Haynes *et al.* 2005). The broadly neutralizing mAb 2G12 was found to bind to the heavily glycosylated, functionally “silent” face of gp120 with an affinity similar to that of a protein-protein interaction. Crystal structure determination revealed that by interdigitation of the Fab-domains (V_H -domain swapping) an increased antigen-binding surface is generated allowing for multivalent interaction with conserved mannose structures (Calarese *et al.* 2005).

Affinity measurement using plasmon surface resonance is widely used to characterize the binding ability of antibodies to their targets and in many cases a correlation can be observed with their biological activity. For virus neutralization, however, it has repeatedly been reported that no such correlation was found (Wu *et al.* 2005, Bakker *et al.* 2005). A possible explanation could be that a low affinity to a functionally important neutralizing epitope results in a higher potency of the mAb compared with a higher affinity to a less important epitope. This may be illustrated with the SARS coronavirus neutralizing mAbs CR3014 and CR3022, which we

isolated from a naïve and an immune library, respectively (van den Brink *et al.* 2005, ter Meulen, unpublished). Both block binding of SARS-CoV to its cellular receptor ACE2 on Vero cells, as shown in FACS-experiments using the recombinant expressed S1 subunit of the spike glycoprotein, which contains the receptor binding domain (RBD). Both antibodies bind to the RBD in a non-competing fashion, as shown in competitive ELISA. However, CR3014 has a rather low affinity to recombinant S1 ($K_D = 27$ nM) but a reasonable potency (7.6 µg/ml for 100% neutralization), whereas CR3022 has a high affinity (300 pM) but lower potency (50 µg/ml). Obviously the mAbs recognize either two different neutralizing epitopes on the RBD, or one binds to another epitope in the S1 subunit thereby inducing a conformational change in the RBD (allosteric effect) or possibly blocks interaction with a putative co-receptor of SARS-CoV.

Therefore we find it preferable to characterize all antibodies first in a rapid screening assay for *in vitro* neutralization, and thereafter define the biochemical characteristics of the most potent ones. However, the neutralizing activity of an antibody may involve factors not present in standard neutralization, i.e. cell culture protection assay, such as complement fixation. Furthermore, if the *in vivo* neutralizing activity involves antibody dependent cellular cytotoxicity (ADCC) or perhaps signalling to infected cells, mAbs with these properties will also be missed. It has been observed that *in vitro* potency may not be predictive of *in vivo* potency, and even non-neutralizing antibodies may confer *in vivo* protection (Iacono-Conors *et al.* 1996, Griffin *et al.* 1997, Mozdzanowska *et al.* 1997). Since high-throughput screening of antibodies in animals is not feasible, there is a need for the development of additional *in vitro* assays for measuring antiviral antibody functions other than blocking of attachment and entry.

Potent neutralizing mAbs will then be further characterized with respect to their target, normally a viral attachment protein on the surface of the virion, which can be identified by Western blot or immunoprecipitation. Binding to known antigenic domains can conveniently be tested in a competition ELISA by blocking binding sites with antibodies whose binding sites are known. If the epitopes recognized are linear, they can be mapped by pepscan analysis with overlapping peptides corresponding to the amino acid (aa) sequence of the protein and by subsequent alanine scan to identify the aa critical for binding. Conformational domains are mapped by sequencing escape variants of the virus generated with subneutralizing antibody concentrations. If the crystal structure of the target protein is known, the 3D localisation of the amino acids critical for neutralization can be determined and the molecular mechanism of neutralisation may be speculated upon (Daffis *et al.* 2005). These neutralization variants are also important to understand the likelihood and mechanism of immune escape induced by a

mAb, and how to prevent it by combining two neutralizing mAbs targeting different neutralizing epitopes.

Preclinical development of a mAb requires demonstration of efficacy and safety in animal models. While infected animals replicate many viruses to some titer, they often do not develop a clinical state comparable to the human disease and show a different pathology. In order to limit the number of animals used and unequivocally demonstrate efficacy of an antibody or vaccine, 100% lethal animal test systems have been developed, often based on suckling mice. These systems are convenient to test pre- or post-exposure prophylaxis with a mAb, especially if there is a clear dose-response of virus challenge and mortality as well as mAb concentration and protection. Unfortunately, for many viral infections no small animal models are available which would generate a clinical and pathological state more closely resembling human disease. In case of chronic viral infections such as HIV, hepatitis B and hepatitis C, non-human primates (NHP) or chimpanzees are the most meaningful animal models, which makes evaluation of antivirals extremely costly and time consuming.

The most extensive studies in NHP were performed with anti-HIV mAbs, reviewed by Ferrantelli and Ruprecht 2002. Passive immunization with neutralizing human mAbs – used alone or in combination with other monoclonal or polyclonal neutralizing antibodies – completely prevented infection in some adult animals challenged intravenously or intravaginally, and in neonatal monkeys challenged orally. Most animals that did become infected had low viral RNA loads and were protected from challenge virus-induced acute disease. In a recent study, a cocktail of three human mAbs with potent cross-clade and cross-group HIV-neutralizing activity was given to neonatal rhesus macaques one hour and again eight days after oral HIV challenge; all animals remained virus negative for > 1 year (Ferrantelli *et al.* 2004).

3. CLINICAL TRIALS WITH ANTIVIRAL MONOCLONAL ANTIBODIES

Current clinical trials with mAbs are targeting well defined groups of patients, in which the medical and economic consequences of unchecked viral replication justify the comparatively high costs of a mAb prophylaxis.

Endogenous reinfection of an orthotopic liver transplant with hepatitis B or C presents a major medical problem, since the virus remains in several other body compartments following removal of the infected liver. Without

treatment, reinfection of the transplanted liver occurs rapidly resulting in progressive disease, graft failure, and death. Life-long prophylactic treatment is therefore necessary. A mixture of two fully human monoclonal antibodies, directed against different epitopes of hepatitis B surface antigen (HBsAg) and binding to all major HBV subtypes, is presently being studied in a Phase IIb clinical trial in liver transplant patients (XTL Biopharmaceuticals, Israel). In an earlier phase I clinical study, a total of 27 chronic HBV patients were enrolled. In part A of the study 15 patients in 5 cohorts received a single intravenous infusion of antibodies with doses ranging from 0.26 mg (260 IU) to 40 mg (40,000 IU). All patients completed 16 weeks of follow-up. In the second part of the study (part B), 12 patients in 4 cohorts received 4 weekly infusions of 10, 20, 40, or 80 mg each of the mAb cocktail and were followed for 4 additional weeks. Patients administered doses at an Ab:Ag molar ratio of 1:2 to 1:20 showed a rapid and significant decrease in HBsAg to undetectable levels, with a corresponding reduction of HBV-DNA levels. In part B, the mAbs induced a significant reduction in both HBsAg and HBV-DNA levels repeatedly after administration (Galun *et al.* 2002).

The same company is conducting a phase Ia/b clinical trial with a combination of two anti-HCV-E2 mAbs to prevent hepatitis C re-infection following a liver transplant and for the treatment of chronic HCV. mAb68 and mAb65 immunoprecipitate viral particles from patients' sera infected with different HCV genotypes and incubation of an infectious human serum with Ab68 or Ab65 prevented the serum's ability to infect human liver cells and human liver tissue. A Phase IIa Clinical Trial was performed with Ab68 following liver transplant, which demonstrated the safety and tolerability of the mAb up to 240mg dosed for 12 weeks. The 120mg and 240mg dose groups had a significantly greater reduction in viral load than the placebo group during the first week when dosed daily. This effect was less evident when dosed less frequently than daily. It is thought that a combination of two antibodies that bind to different epitopes is essential to provide broad coverage of virus quasispecies, and to minimize the probability of escape from therapy.

To prevent CMV infection after allogeneic hematopoietic stem cell transplantation, a mAb (MSL-109) specific to the cytomegalovirus (CMV) glycoprotein H with high neutralizing capacity was evaluated in a prospective, randomized, double-blind study (Boeckh *et al.* 2001). Allogeneic hematopoietic stem cell transplantation (HSCT) recipients with positive donor and/or recipient serology for CMV before transplantation received either 60 or 15 mg/kg MSL-109, or placebo intravenously every 2 weeks from day -1 until day 84 after transplantation. CMV pp65 antigenemia, CMV-DNA load in plasma, and viremia by culture were tested weekly. Primary end points were development of pp65 antigenemia at any

level and/or viremia for which ganciclovir was given. There was no statistically significant difference in CMV pp65 antigenemia or viremia among patients in the mAb and the placebo group.

In two small phase I studies, two human mAbs and one humanized anti-HIV mAb directed against gp120 and gp41 were reported to transiently lower the viral load in five of seven and three of four patients, respectively (Stiegler *et al.* 2002, Dezube *et al.* 2004). These initially encouraging results led to a recent proof-of-concept study designed to mimic therapeutic vaccination by administering a cocktail of three neutralizing human mAbs (2G12, 2F5, 4E10), interrupting antiretroviral therapy (ART) and measuring the level of viral rebound (Trkola *et al.* 2005). Eight chronically and six acutely HIV-1 infected patients, all with undetectable levels of viremia while on ART, received multiple infusions of the mAbs over an 11-week period. ART was discontinued one day after commencing the antibody treatment. The HIV strains harboured by all patients were shown to be highly sensitive to neutralization with at least two mAbs. No side effects of the mAb therapy were observed, despite 2F5 and 4E10 having been shown to be cross-reactive with cardiolipin (Haynes *et al.* 2005). Viral rebound to pre-ART plasma levels occurred without apparent delay in six chronic and two acute patients. Rebound was delayed in the remaining six patients, but then rose to pre-ART levels in one acute and one chronic subject in the presence of high plasma concentrations of the mAbs. In two acute patients, rebound peaked 2-4 logs below pre-ART levels and remained low after the mAbs were cleared from the circulation. One subject had no detectable viral rebound. Surprisingly, most of the effect of the treatment could be attributed to mAb 2G12 only. Patients with a delay in rebound had very high plasma levels of this mAb with on average 400 times its IC₉₀ and more than double the highest effective dose for 2F5 and 4E10 in any patient, probably due to the longer plasma half-life of 2G12. Furthermore, 2G12 was the only antibody that rebound viruses escaped, while remaining sensitive to 2F5 and 4E10. It was therefore speculated that the plasma concentrations achieved for 2F5 and 4E10 were below a crucial threshold needed to control the virus. In conclusion, this study showed that control of HIV by neutralizing mAbs may be possible but will require high concentrations of combinations of antibodies capable of preventing immune escape or combinations with other antiviral drugs reducing replication of the virus.

The combination of mAbs with antiviral drugs in clinical trials is discussed below.

4. STRATEGIES TO IMPROVE ANTIVIRAL MONOCLONAL ANTIBODIES

Based on experimental data and results from clinical trials, prophylaxis and therapy of viral infections with mAbs is feasible if the breadth of protection against viral strains is sufficient, generation of immune escape variants is prevented and the mAbs are of such high potency that they can be administered at low, economically feasible doses.

4.1 Increasing potency through affinity maturation or altering the avidity

It has been shown that increased binding ability to a critical viral epitope results in greater neutralizing potency of antiviral monoclonal antibodies (Johnson *et al.* 1999). Increased binding may result from increased affinity, as determined by the association (k_{on}) and dissociation (k_{off}) rates in plasmon surface resonance (BIAcore) measurements, or from increased avidity, due to multivalent binding (Alfthan 1998).

The affinities of antibody fragments derived from naïve repertoires of $\sim 10^8$ immunoglobulin genes appear to be in the micromolar range, characteristic for primary immune responses (Griffiths *et al.* 1993). *In vivo*, the primary immune response is further improved via a process of mutations and selections called affinity maturation. This process can be mimicked *in vitro* to optimize the antibodies selected by phage display (Chowdhury *et al.* 1999). To improve the affinity, the six CDRs of the heavy and light chains fragments can be subjected to mutagenesis or chain shuffling (recombining a V_{H} or V_{L} chain with a library of partner chains), followed by phage based selection (Boder *et al.* 2000, Yang *et al.* 1995, Wu *et al.* 2005, Thompson *et al.* 1996). The affinities achieved *in vitro* have been in the nanomolar or picomolar range, which is comparable to affinities of antibodies isolated from immune libraries.

Although antibody affinity is mediated by both k_{on} and k_{off} , the greatest affinity improvements through *in vitro* maturation have been achieved by reducing k_{off} . Selected or combinatorial point mutations in the CDRs have been reported to reduce k_{off} up to 10-fold and 100-fold respectively, and were reported to improve their *in vitro* HIV neutralization potency (Yang *et al.* 1995, Thompson *et al.* 1996). However, as was recently shown for antibody variants derived from the anti-RSV antibody Palivizumab, k_{on} and k_{off} may have differential effects on the neutralizing potency of F_{ab} -fragments and full IgGs, respectively. A modest four to five-fold increased association rate resulted in a dramatic 20-fold increase in potency, whereas

even a 100-fold decrease in dissociation rate did not significantly increase potency (Wu 2005).

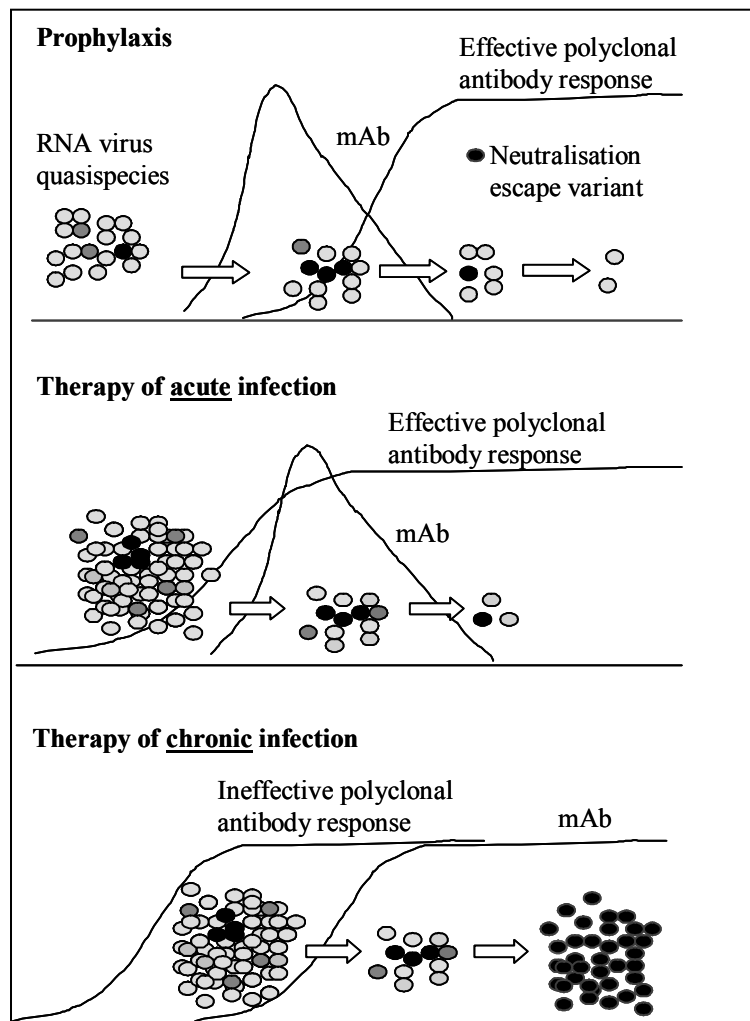


Figure 2. Immune escape induced by antiviral mAbs is less likely to occur in pre- and postexposure prophylaxis and therapy of acute viral infections, because of the effective polyclonal immune and T-cell response. In contrast, in chronic infections the virus reservoir is not eradicated by the mAb and the ineffective polyclonal antibody response will not control escape variants.

The authors suggest that a fast association rate may enable the mAb to neutralize the virus before it infects susceptible cells.

Expressing mAbs as IgM molecules has also been shown to increase the *in vitro* neutralizing potency of anti-HIV mAbs, possibly mediated both by increased avidity and by increased complement fixation (Wolbank *et al.* 2003). However, the IgM-format of recombinant antibodies is currently more difficult to produce and pharmacologically less well evaluated than the standard IgG1 format. To this end, novel antibody formats based on expressing antigen-binding fragments as multivalent molecules (triabodies, tetrabodies) are very interesting recent developments (Hollinger 2005).

4.2 Increasing potency, breadth of protection and preventing immune escape through mixing of non-competing mAbs

Due to their exquisite epitope specificity mAbs may not cover all clinically relevant strains of a virus or rapidly select neutralization escape variants from the quasispecies which RNAs viruses tend to form. However, both in prophylaxis and early therapy of acute infections immune escape is unlikely to occur because the mAb is the first line of defence after which an effective polyclonal immune response should eliminate any escape variants (figure 2). In chronic infections, immune escape variants will be selected by the mAb unless the virus reservoir eradicated.

For acute infections, the immune escape problem can be solved by combining two neutralizing, non-competing mAbs as we have shown for rabies virus. Rabies virus infection in man is 100% fatal, unless the vaccine and human rabies immune globulin (HRIG) are administered immediately after exposure. The disease is most frequently caused by transmission of one of several serotypes of Lyssa virus, genotype 1 through the bite of an infected, rabid canine. Several additional animal species, notably bats, harbour other of the 12 currently known genotypes of Lyssa virus (rabies and rabies-like viruses), some of which have caused disease in man. Therefore, any mAb used for replacement of HRIG must provide the same breadth of protection, most importantly against rabies viruses transmitted by rabid animals ("street viruses"). In addition, there is a hypothetical risk of selecting a viral neutralization escape variant under selective pressure of the mAb, despite only limited replication of rabies virus at the site of inoculation.

To address these problems, we identified and characterized two non-competing human mAbs, binding to antigenic sites I and III of the rabies virus glycoprotein. Their epitopes were mapped by generating neutralization escape variants, which could be neutralized by the respective other mAb.

Furthermore, the mAb combo was shown to have the same breadth of protection as HRIG and high *in vitro* and *in vivo* potency (additive effect). Analysis of the linear epitopes of these antibodies revealed that the majority of natural rabies virus isolates contain both intact epitopes and the few remaining strains contain at least one of the two (Bakker *et al.* 2005). These mAb offer for the first time the possibility to replace HRIG with a safe, potent and cheap recombinant product available in unlimited quantity.

In chronic infections, escape from neutralizing antibodies has readily been observed in humans. Neutralizing antibodies can protect against challenge with HIV-1 *in vivo* if present at appropriate concentrations at the time of virus challenge, but their role in the control of established infection is unclear. Autologous neutralizing antibodies (Nab) can be detected as early as 52 days after detection of HIV-specific antibodies. The viral inhibitory activity of Nab results in complete replacement of neutralization-sensitive virus by successive populations of resistant virus, involving primarily changes in N-linked glycosylation (Wei *et al.* 2003). In a phase I study with a cocktail of two neutralizing mAbs (2F5, 4E10, 2G12), immune escape was observed with one of the mAbs (Trkola *et al.* 2005). Experimentally, even high serum concentrations of neutralizing monoclonal antibodies, either singly or as a cocktail, have little sustained effect on viral load in established HIV-1 infection in hu-PBL-SCID mice. In some instances, virus replication of neutralization-sensitive virus continues even in the presence of high levels of neutralizing antibody. In most instances, neutralization escape occurs in a few days, even from a cocktail of three antibodies that recognize distinct epitopes (Poignard 1999). The authors concluded that humoral immunity is unlikely to play a significant role in the control of established HIV-1 infection in humans. Similar observations have been made in hepatitis B and C (Rehermann and Nascimbeni 2005). It is therefore questionable whether the action of neutralizing mAbs can be improved such that the immune escape problem in chronic infection can be controlled by antibody alone.

In certain instances, combining mAbs may not only increase the breadth of protection, but also lead to an increase of the *in vitro* and *in vivo* potency of the mixture disproportionately to the individual potencies of the antibodies (synergistic action), as has been shown for combinations of two, three or four mAbs directed against different epitopes on the HIV-1 envelope glycoprotein (Li *et al.* 1998, Zwick *et al.* 2001, Xu *et al.* 2001). We have observed synergistic neutralization of SARS-CoV. Human mAb CR3014 isolated from a naïve antibody phage library and non-competing mAb CR022 isolated from an immune library were combined in equimolar ratios

and the concentration required for 100% and 50% neutralization of SARS-CoV was determined. Applying a mathematical model based on the mass-action law principle (Chou and Talalay 1984) revealed that CR3014 and CR3022 neutralize SARS-CoV synergistically *in vitro*, with dose reduction indices of 3 and 20, respectively. Mechanistically, cooperative binding of mAbs may induce conformational changes in the antigen thereby altering affinities (allosteric effect), lead to intermolecular interaction of bivalent antibodies or result in interaction of the Fc regions of antibodies brought into close contact (Klonisch *et al.* 1996). As determined by ELISA, CR3014 and CR3022 bind simultaneously to the RBD of the S1 subunit of the glycoprotein spike of SARS-CoV. However, measuring separate, sequential and simultaneous binding of the mAbs in Biacore did not reveal enhanced affinity of the antibody mixture over the individual affinities, especially for CR3022. Neutralization escape variants generated with one anti-SARS-CoV mAb were effectively neutralized by the other. When administered prophylactically to ferrets before intratracheal challenge with SARS-CoV, mAb CR3014 prevented lung pathology due to viral replication and abolished shedding of the virus in pharyngeal secretions at a dose of 10mg/kg (ter Meulen *et al.* 2004). The synergistic action of the mAbs may allow for a lower total combined antibody dose to reach the same potency, which increases the economic attractiveness of a potential product for immunoprophylaxis of SARS.

4.3. Synergy of antibodies and antiviral drugs

Few experimental studies have addressed the potential synergy of neutralizing antibodies and antiviral drugs, however, data from clinical studies combining immune serum and antivirals may allow some conclusions.

Orthotopic liver transplantation for hepatitis B virus (HBV) infection was limited until recently by poor graft and patient outcomes caused by recurrent HBV. Long-term immunoprophylaxis with hepatitis B immune globulin (HBIG) dramatically improved post-transplantation survival, but recurrent HBV still occurred in up to 36% of the recipients due to the occurrence of immune escape mutants. More recently, combination HBIG and lamivudine has been shown to effectively prevent HBV recurrence in patients post-transplantation. However, optimum doses and duration for these drugs are not yet clear. The combination of lamivudine 300 mg/day and low-dose HBIG was reported to prevent post-transplantation recurrence of hepatitis B, even in the presence of viral replication in the pre-transplant period (Karasu

et al. 2004). Furthermore, combination therapy resulted in a cost-effective strategy with an average cost-effectiveness ratio of \$252,111/recurrence prevented compared with \$362,570/recurrence prevented in the monotherapy strategy. It was concluded that combination prophylaxis with HBIG and lamivudine is highly effective in preventing recurrent HBV, may protect against the emergence of resistant mutants, and is significantly more cost-effective than HBIG monotherapy with its associated rate of recurrent HBV (Han *et al.* 2000). However, in low-risk liver transplant patients who were HBsAg-positive/HBV DNA-negative before transplantation and had received HBIG for at least 6 months without HBV recurrence, lamivudine alone was effective for prevention of HBV recurrence (Naoumov *et al.* 2001). Another study also concluded that lamivudine monotherapy after a short course of lamivudine and HBIG was equally as efficacious in preventing HBV recurrence as HBIG plus lamivudine during the first 18 months after liver transplantation (Buti *et al.* 2003).

In seronegative heart transplant recipients receiving an allograft from seropositive donors, double CMV prophylaxis consisting of CMV hyperimmune globulin and ganciclovir was shown to abolish CMV death and to prevent CMV disease (Bonaros *et al.* 2004). In contrast to these findings, a human monoclonal antibody directed against CMV gH for the treatment of newly diagnosed CMV retinitis in AIDS patients did not consistently produce clinical improvement (Borucki *et al.* 2004).

A synergistic effect of combining immune serum and the guanosine analogue ribavirin has been shown clinically for therapy of measles pneumonia (Barnard 2004, Stogner *et al.* 1993). In a mouse model of lymphocytic choriomeningitis (LCMV) infection, monoclonal antibody expression in transgenic mice alone was unable to prevent the establishment of persistent infection due to generation of neutralization escape variants. Daily administration of ribavirin resulted in elimination of the virus after two weeks and virus isolated at earlier time points was shown to remain sensitive to mAb neutralization (Seiler *et al.* 2000). It was concluded that the main effect of ribavirin was reduction of viral replication efficiency, which rendered the appearance of antibody escape variants less frequent and lowered viral titers sufficiently so that CTLs were able to control the virus. In agreement with this study, virus-specific immune plasma was shown to enhance the beneficial effect of ribavirin on primate survival and virus replication after infection with another member of the arenavirus family, the Lassa virus (Jahrling *et al.* 1984).

5. CONCLUSION AND OUTLOOK

Many viral infections are currently prevented by passive prophylaxis with human immune serum, with only some being amenable to serum therapy. The state of monoclonal antibody technology now makes it possible to replace human sera by custom-tailored human monoclonal antibodies, thereby solving the safety, cost and supply problems inherent in blood products derived from human donors. However, only a few biotech companies have currently taken mAbs for HIV, hepatitis B, C, CMV and RSV infection in preclinical or clinical development. Economically, monoclonal antibodies are seen as less attractive antivirals than small molecule drugs due to the approximately two-fold increase in cost and time required to produce clinical-grade material. Scientifically, the possibly limited breadth of protection, generation of immune escape variants and uncertain clinical efficacy in case of established acute or chronic infection are perceived as major obstacles to the development of mAb products for control of viral infections.

We speculate that several of the antiviral mAbs which have produced unsatisfactory results in preclinical or clinical studies were not optimally selected. Based on our own data and published evidence we therefore propose a paradigm shift, predicting that a broad-scale screening for synergistic action of antibody cocktails as stand-alone or in combination with antivirals may solve many of the above outlined problems. Crucial to the discovery process of highly potent antiviral mAbs is a high-throughput screening based on *in vitro* or ideally *in vivo* neutralization, which should systematically investigate the possible neutralizing synergy of non-competing antibodies. Synergy of the mAbs with antivirals could also be included in an early screening step. If the potency of an individual antibody needs to be increased, selection for an improved k_{on} should be performed. Novel antibody formats based on multimerization of antigen-binding fragments may lead to molecules with high avidity and increased neutralizing potency.

Importantly, the state of recombinant antibody technology offers a unique opportunity to rapidly counteract epidemic threats of newly emerging diseases. It took less than a year to discover human mAbs which protect against SARS-coronavirus in different animal models and could be used for human ring vaccination in case of an outbreak or a bioterror attack. Avian influenza may represent another attractive target for passive immunization, if mAbs cross-reactive with the current Asian H5 strains can be found.

Economically, the increased production costs of mAbs and the longer production time compared to conventional small-molecules are set-off by a faster and more predictable clinical trajectory. The approval success rate for chimeric/humanized antibodies is currently 18-29% compared to 11% for new chemical entities (NEC) as a whole (Reichert 2005). Both academia and industry should therefore revisit the opportunity of using monoclonal antibodies for prophylaxis and therapy of viral infections.

ACKNOWLEDGEMENTS

We would like to thank all the colleagues from Crucell who have helped in many invaluable ways in the production of this chapter, in particular the Antibody Discovery group: J de Kruif, ABH Bakker, M Throsby, WE Marissen, RA Kramer, EN van den Brink, AQ Bakker, M Clijsters-van der Horst, TJ Visser, M Jongeneelen, S Thijssse, F Cox and M de Jong. We thank G. Turner for editing of the manuscript.

REFERENCES

- Alfthan K, 1998, Surface plasmon resonance biosensors as a tool in antibody engineering. *Biosensors & Bioelectronics* **13**: 653-663.
- Arakelov S, Gorny MK, Williams C, Riggan CH, Brady F, Collett MS, Zolla-Pazner S, 1993, Generation of neutralizing anti-B19 parvovirus human monoclonal antibodies from patients infected with human immunodeficiency virus. *J. Infect. Dis.* **168**: 580-585.
- Baker M, 2005, Upping the ante on antibodies. *Nat. Biotechnol.* **23**: 1065-1072.
- Bakker AB, Marissen WE, Kramer RA, Rice AB, Weldon WC, Niezgodka M, Hanlon CA, Thijssse S, Backus HH, de Kruif J, Dietzschold B, Rupprecht CE, Goudsmit J, 2005, Novel human monoclonal antibody combination effectively neutralizing natural rabies virus variants and individual *in vitro* escape mutants. *J. Virol.* **79**: 9062-9068.
- Barnard DL, 2004, Inhibitors of measles virus. *Antivir. Chem. Chemother.* **15**: 111-119.
- Bjorling E, von Garrelts E, Morner A, Ehnlund M, Persson MA, 1999, Human neutralizing human immunodeficiency virus type 2-specific Fab molecules generated by phage display. *J. Gen. Virol.* **80**: 1987-1993.
- Boder ET, Midelfort KS, Wittrup KD, 2000, Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc. Natl. Acad. Sci. USA* **97**: 10701-10705.
- Boeckh M, Bowden RA, Storer B, Chao NJ, Spielberger R, Tierney DK, Gallez-Hawkins G, Cunningham T, Blume KG, Levitt D, Zaia JA, 2001, Randomized, placebo-controlled, double-blind study of a cytomegalovirus-specific monoclonal antibody (MSL-109) for

- prevention of cytomegalovirus infection after allogeneic hematopoietic stem cell transplantation. *Biol. Blood Marrow Transplant.* **7**: 343-51.
- Bonaros NE, Kocher A, Dunkler D, Grimm M, Zuckermann A, Ankersmit J, Ehrlich M, Wolner E, Laufer G, 2004, Comparison of combined prophylaxis of cytomegalovirus hyperimmune globulin plus ganciclovir versus cytomegalovirus hyperimmune globulin alone in high-risk heart transplant recipients. *Transplantation* **77**: 890-7.
- Borucki MJ, Spritzler J, Asmuth DM, Gnann J, Hirsch MS, Nokta M, Aweeka F, Nadler PI, Sattler F, Alston B, Nevin TT, Owens S, Waterman K, Hubbard L, Caliendo A, Pollard RB; AACTG 266 Team, 2004, A phase II, double-masked, randomized, placebo-controlled evaluation of a human monoclonal anti-Cytomegalovirus antibody (MSL-109) in combination with standard therapy versus standard therapy alone in the treatment of AIDS patients with Cytomegalovirus retinitis. *Antiviral Res.* **64**:103-11.
- Buti M, Mas A, Prieto M, Casafont F, Gonzalez A, Miras M, Herrero JI, Jordi R, Cruz de Castro E, Garcia-Rey C, 2003, A randomized study comparing lamivudine monotherapy after a short course of hepatitis B immune globulin (HBIG) and lamivudine with long-term lamivudine plus HBIG in the prevention of hepatitis B virus recurrence after liver transplantation. *J. Hepatol.* **38**: 811-817.
- Calarese DA, Lee HK, Huang CY, Best MD, Astronomo RD, Stanfield RL, Katinger H, Burton DR, Wong CH, Wilson IA, 2005, Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. *Proc. Natl. Acad. Sci. USA* **102**: 13372-13377.
- Casadevall A, Dadachova E, Pirofski LA, 2004, Passive antibody therapy for infectious diseases. *Nat. Rev. Microbiol.* **2**: 695-703.
- Chou TC, Talalay P, 1984, Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**: 27-55.
- Chowdhury PS, Pastan I, 1999, Improving antibody affinity by mimicking somatic hypermutation in vitro. *Nat. Biotechnol.* **17**: 568-572.
- Daffis S, Kontermann RE, Korimbocus J, Zeller H, Klenk HD, ter Meulen J, 2005, Antibody responses against wild-type yellow fever virus and the 17D vaccine strain: characterization with human monoclonal antibody fragments and neutralization escape variants. *Virology* **337**: 262-272.
- de Carvalho Nicacio C, Lundkvist A, Sjolander KB, Plyusnin A, Salonen EM, Bjorling E, 2000, A neutralizing recombinant human antibody Fab fragment against Puumala hantavirus. *J. Med. Virol.* **60**: 446-454.
- de Carvalho Nicacio C, Williamson RA, Parren PW, Lundkvist A, Burton DR, Bjorling E, 2002, Neutralizing human Fab fragments against measles virus recovered by phage display. *J. Virol.* **76**: 251-258.
- de Kruijf J, Boel E, Logtenberg T, 1995, Selection and application of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. *J. Mol Biol.* **248**: 97-105.
- Dezube BJ, Doweiko JP, Prope JA, Conway B, Hwang L, Terada M, Leece BA, Ohno T, Mastro RA, 2004, Monoclonal antibody hNM01 in HIV-infected patients: a phase 1 study. *J. Clin. Virol.* **31S**: S45-S47.
- Ennsle K, Kurrle R, Kohler R, Muller H, Kanzy EJ, Hilfenhaus J, Seiler FR, 1991, A rabies-specific human monoclonal antibody that protects mice against lethal rabies. *Hybridoma* **10**: 547-556.

- Eren R, Ilan E, Nussbaum O, Lubin I, Terkieltaub D, Arazi Y, Ben-Moshe O, Kitchinzky A, Berr S, Gopher J, Zauberma A, Galun E, Shouval D, Daudi N, Eid A, Jurim O, Magnius LO, Hammas B, Reisner Y, Dagan S, 2000, Preclinical evaluation of two human anti-hepatitis B virus (HBV) monoclonal antibodies in the HBV-trimera mouse model and in HBV chronic carrier chimpanzees. *Hepatology* **32**: 588-596.
- Eren R, Lubin I, Terkieltaub D, Ben-Moshe O, Zauberma A, Uhlmann R, Tzahor T, Moss S, Ilan E, Shouval D, Galun E, Daudi N, Marcus H, Reisner Y, Dagan S, 1998, Human monoclonal antibodies specific to hepatitis B virus generated in a human/mouse radiation chimera: the Trimera system. *Immunology* **93**: 154-161.
- Ferrantelli F, Ruprecht RM (2002). Neutralizing antibodies against HIV – back in the major leagues? *Curr Opin Immunol.* **14**: 495-502.
- Ferrantelli F, Rasmussen RA, Buckley KA, Li PL, Wang T, Montefiori DC, Katinger H, Stiegler G, Anderson DC, McClure HM, Ruprecht RM, 2004, Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies. *J. Infect. Dis.* **189**: 2167-2173.
- Galun E, Eren R, Safadi R, Ashour Y, Terrault N, Keeffe EB, Matot E, Mizrahi S, Terkieltaub D, Zohar M, Lubin I, Gopher J, Shouval D, Dagan S, 2002, Clinical evaluation (phase I) of a combination of two human monoclonal antibodies to HBV: safety and antiviral properties. *Hepatology.* **35**: 673-679.
- Goncalvez AP, Men R, Wernly C, Purcell RH, Lai CJ, 2004, Chimpanzee Fab fragments and a derived humanized immunoglobulin G1 antibody that efficiently cross-neutralize dengue type 1 and type 2 viruses. *J.Virol.* **78**:12910-12918.
- Greenough TC, Babcock GJ, Roberts A, Hernandez HJ, Thomas WD Jr, Coccia JA, Graziano RF, Srinivasan M, Lowy I, Finberg RW, Subbarao K, Vogel L, Somasundaran M, Luzuriaga K, Sullivan JL, Ambrosino DM, 2005, Development and characterization of a severe acute respiratory syndrome-associated coronavirus-neutralizing human monoclonal antibody that provides effective immunoprophylaxis in mice. *J. Infect. Dis.* **191**: 507-514.
- Griffiths AD, Malmqvist M, Marks JD, Bye JM, Embleton MJ, McCafferty J, Baier M, Holliger KP, Gorick BD, Hughes-Jones NC, Hoogenboom HR, Winter G, 1993, Human anti-self antibodies with high specificity from phage display libraries. *EMBO* **12**: 725-734.
- Griffin D, Levine B, Tyor W, Ubol S, Despres P, 1997, The role of antibody in recovery from alphavirus encephalitis. *Immunol. Rev.* **159**: 155-161.
- Habersetzer F, Fournillier A, Dubuisson J, Rosa D, Abrignani S, Wychowski C, Nakano I, Trepo C, Desgranges C, Inchauspe G, 1998, Characterization of human monoclonal antibodies specific to the hepatitis C virus glycoprotein E2 with *in vitro* binding neutralization properties. *Virology* **249**: 32-41.
- Han SH, Ofman J, Holt C, King K, Kunder G, Chen P, Dawson S, Goldstein L, Yersiz H, Farmer DG, Ghobrial RM, Busuttill RW, Martin P, 2000, An efficacy and cost-effectiveness analysis of combination hepatitis B immunoglobulin and lamivudine to prevent recurrent hepatitis B after orthotopic liver transplantation compared with hepatitis B immune globulin monotherapy. *Liver Transpl.* **6**: 741-8.
- Hanlon CA, DeMattos CA, DeMattos CC, Niezgodka M, Hooper DC, Koprowski H, Notkins A, Rupprecht CE, 2001, Experimental utility of rabies virus-neutralizing human monoclonal antibodies in post-exposure prophylaxis. *Vaccine* **19**: 3834-42.
- Haynes BF, Fleming J, St Clair EW, Katinger H, Stiegler G, Kunert R, Robinson J, Searce RM, Plonk K, Staats HF, Ortel TL, Liao HX, Alam SM, 2005, Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science* **2308**: 1906-1908.

- He Y, Honnen WJ, Krachmarov CP, Burkhart M, Kayman SC, Corvalan J, Pinter A, 2002, Efficient isolation of novel human monoclonal antibodies with neutralizing activity against HIV-1 from transgenic mice expressing human Ig loci. *J. Immunol.* **169**: 595-605.
- Higo-Moriguchi K, Akahori Y, Iba Y, Kurosawa Y, Taniguchi K, 2004, Isolation of human monoclonal antibodies that neutralize human rotavirus. *J. Virol.* **78**: 3325-3332.
- Hollinger P, Hudson PJ, 2005, Engineered antibody fragments and the rise of single domains. *Nat. Biotechnol.* **9**: 1126-1136.
- Hong HJ, Ryu CJ, Hur H, Kim S, Oh HK, Oh MS, Park SY, 2004, In vivo neutralization of hepatitis B virus infection by an anti-preS1 humanized antibody in chimpanzees. *Virology* **318**: 134-141.
- Hoogenboom H, 2005, Selecting and screening recombinant antibody libraries. *Nature Biotechnology* **23**: 1105-1116.
- Hoogenboom HR, de Bruine AP, Hufton SE, Hoet RM, Arends JW, Roovers RC, 1998, Antibody phage display technology and its applications. *Immunotechnology* **4**: 1-20.
- Iacono-Connors LC, Smith JF, Ksiazek TG, Kelley CL, Schmaljohn CS, 1996, Characterization of Langkat virus antigenic determinants defined by monoclonal antibodies to E, NS1 and preM and identification of a protective, non-neutralizing preM-specific monoclonal antibody. *Virus Res.* **43**: 125-136.
- Jahrling PB, Peters CJ, Stephen EL, 1984, Enhanced treatment of Lassa fever by immune plasma combined with ribavirin in cynomolgous monkeys. *J. Infect. Dis.* **149**: 420-427.
- Jakovovits A., 1998, Production and selection of antigen-specific fully human monoclonal antibodies from mice engineered with human Ig loci. *Adv. Drug. Deliv. Rev.* **31**: 33-42.
- Johnson S, Griego SD, Pfarr DS, Doyle ML, Woods R, Carlin D, Prince GA, Koenig S, Young JF, Dillon SB, 1999, A direct comparison of the activities of two humanized respiratory syncytial virus monoclonal antibodies: MEDI-493 and RSHZ19. *J. Infect. Dis.* **180**: 35-40.
- Johnson S, Oliver C, Prince GA, Hemming VG, Pfarr DS, Wang SC, Dormitzer M, O'Grady J, Koenig S, Tamura JK, Woods R, Bansal G, Couchenour D, Tsao E, Hall WC, Young JF, 1997, Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. *J. Infect. Dis.* **176**: 1215-1224.
- Karasu Z, Ozacar T, Akyildiz M, Demirbas T, Arikan C, Kobat A, Akarca U, Ersoz G, Gunsar F, Batur Y, Kilic M, Tokat Y, 2004, Low-dose hepatitis B immune globulin and higher-dose lamivudine combination to prevent hepatitis B virus recurrence after liver transplantation. *Antivir Ther.* **9**: 921-7.
- Kausmally L, Waalen K, Lobersli I, Hvattum E, Berntsen G, Michaelsen TE, Brekke OH, 2004, Neutralizing human antibodies to varicella-zoster virus (VZV) derived from a VZV patient recombinant antibody library. *J. Gen. Virol.* **85**: 3493-3500.
- Keck ZY, Sung VM, Perkins S, Rowe J, Paul S, Liang TJ, Lai MM, Fong SK, 2004, Human monoclonal antibody to hepatitis C virus E1 glycoprotein that blocks virus attachment and viral infectivity. *J. Virol.* **78**: 7257-7263.
- Kellermann SA, Green LL, 2002, Antibody discovery: the use of transgenic mice to generate human monoclonal antibodies for therapeutics. *Curr. Opin. Biotechnol.* **13**: 593-597.
- Kim SJ, Jang MH, Stapleton JT, Yoon SO, Kim KS, Jeon ES, Hong HJ, 2004, Neutralizing human monoclonal antibodies to hepatitis A virus recovered by phage display. *Virology* **318**: 598-607.
- Klasse PJ and Sattentau QJ, 2002, Occupancy and mechanism in antibody-mediated neutralization of animal viruses. *J. Gen. Virol.* **83**: 2091-2108.
- Klonisch T, Panayotou G, Edwards P, Jackson AM, Berger P, Delves PJ, Lund T, Roitt IM, 1996, Enhancement in antigen binding by a combination of synergy and antibody capture. *Immunology* **89**: 165-171.

- Koch J, Liang M, Queitsch I, Kraus AA, Bautz EK, 2003, Human recombinant neutralizing antibodies against hantaan virus G2 protein. *Virology* **308**: 64-73.
- Koefoed K, Farnaes L, Wang M, Svejgaard A, Burton DR, Ditzel HJ, 2005, Molecular characterization of the circulating anti-HIV-1 gp120-specific B cell repertoire using antibody phage display libraries generated from pre-selected HIV-1 gp120 binding PBLs. *J. Immunol. Methods*. **297**: 187-201.
- Kontermann RE, Dübel S (Eds.), 2001, Antibody engineering, Springer, Heidelberg.
- Kramer RA, Cox F, van der Horst M, van der Oudenrijn S, Res PC, Bia J, Logtenberg T, de Kruif J, 2003, A novel helper phage that improves phage display selection efficiency by preventing the amplification of phages without recombinant protein. *Nucleic Acids Res.* **31**: e59
- Kramer RA, Marissen WE, Goudsmit J, Visser TJ, Clijsters-Van der Horst M, Bakker AQ, de Jong M, Jongeneelen M, Thijsse S, Backus HH, Rice AB, Weldon WC, Rupprecht CE, Dietzschold B, Bakker AB, de Kruif J, 2005, The human antibody repertoire specific for rabies virus glycoprotein as selected from immune libraries. *Eur.J.Immunol.* **35**: 2131-45.
- Li A, Baba TW, Sodroski J, Zolla-Pazner S, Gorny MK, Chou TC, Baba TW, Ruprecht RM, 1998, Synergistic neutralization of simian-human immunodeficiency virus SHIV-vpu+ by triple and quadruple combinations of human monoclonal antibodies and high-titer anti-human immunodeficiency virus type 1 immunoglobulins. *J. Virol.* **72**: 3235-3240.
- Liang M, Mahler M, Koch J, Ji Y, Li D, Schmaljohn C, Bautz EK, 2003, Generation of an HFRS patient-derived neutralizing recombinant antibody to Hantaan virus G1 protein and definition of the neutralizing domain. *J. Med. Virol.* **69**: 99-107.
- Lipovsek D, Pluckthun A, 2004, In-vitro protein evolution by ribosome display and mRNA display. *J. Immunol. Methods* **290**: 51-67.
- Lonberg N, 2005, Human antibodies from transgenic animals. *Nat. Biotechnol.* **23**: 1117-1125.
- Marissen WE, Kramer RA, Rice A, Weldon WC, Niezgodka M, Faber M, Slootstra JW, Meloen RH, Clijsters-van der Horst M, Visser TJ, Jongeneelen M, Thijsse S, Throsby M, de Kruif J, Rupprecht CE, Dietzschold B, Goudsmit J, Bakker AB, 2005, Novel rabies virus-neutralizing epitope recognized by human monoclonal antibody: fine mapping and escape mutant analysis. *J.Virol.* **79**: 4672-8.
- Moulard M, Phogat SK, Shu Y, Labrijn AF, Xiao X, Binley JM, Zhang MY, Sidorov IA, Broder CC, Robinson J, Parren PW, Burton DR, Dimitrov DS, 2002, Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes. *Proc Natl Acad Sci USA* **99**: 6913-6918.
- Mozdzanowska K, Furchner M, Washko G, Mozdzanowski J, Gerhard W, 1997, A pulmonary influenza virus infection in SCID mice can be cured by treatment with hemagglutinin-specific antibodies that display very low virus-neutralizing activity in vitro. *J. Virol.* **71**: 4347-4355.
- Naoumov NV, Lopes AR, Burra P, Caccamo L, Iemmolo RM, de Man RA, Bassendine M, O'Grady JG, Portmann BC, Anschuetz G, Barrett CA, Williams R, Atkins M, 2001, Randomized trial of lamivudine versus hepatitis B immunoglobulin for long-term prophylaxis of hepatitis B recurrence after liver transplantation. *J. Hepatol.* **34**: 888-894.
- Nejatollahi F, Hodgetts SJ, Valley PJ, Burnie JP, 2002, Neutralising human recombinant antibodies to human cytomegalovirus glycoproteins gB and gH. *FEMS Immunol. Med. Microbiol.* **34**: 237-244.
- Parren PW, Burton DR., 2001, The antiviral activity of antibodies in vitro and in vivo. *Adv. Immunol.* **77**: 195-262.

- Parren PW, Geisbert TW, Maruyama T, Jahrling PB, Burton DR., 2002, Pre- and postexposure prophylaxis of Ebola virus infection in an animal model by passive transfer of a neutralizing human antibody. *J. Virol.* **76**: 6408-6412.
- Poignard P, Sabbe R, Picchio GR, Wang M, Gulizia RJ, Katinger H, Parren PW, Mosier DE, Burton DR, 1999, Neutralizing antibodies have limited effects on the control of established HIV-1 infection in vivo. *Immunity* **10**: 431-438.
- Purtscher M, Trkola A, Gruber G, Buchacher A, Predl R, Steindl F, Tauer C, Berger R, Barrett N, Jungbauer A, 1994, A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* **10**:1651-1658.
- Rehermann B, Nascimbeni M, 2005, Immunology of hepatitis B virus and hepatitis C virus infection. *Nat. Rev. Immunol.* **5**: 215-229.
- Reichert JM, Rosenzweig CJ, Faden LB, Dewitz MC, 2005, Monoclonal antibody successes in the clinic. *Nat. Biotechnol.* **9**: 1073-1078.
- Sawyer LA, 2000, Antibodies for the prevention and treatment of viral diseases. *Antiviral Res.* **47**: 57-77.
- Schmaljohn C, Cui Y, Kerby S, Pennock D, Spik K. Production and characterization of human monoclonal antibody Fab fragments to vaccinia virus from a phage-display combinatorial library. *Virology* **258**: 189-200.
- Schofield DJ, Glamann J, Emerson SU, Purcell RH, 2000, Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. *J. Virol.* **74**: 5548-5555.
- Seiler P, Senn BM, Klenermann P, Kalinke U, Hengartner H, Zinkernagel RM, 2000, Additive effect of neutralizing antibody and antiviral drug treatment in preventing virus escape and persistence. *J. Virol.* **74**: 5896-5901.
- Steinitz M, Klein G, Koskimies S, Makel O., 1977, EB virus-induced B lymphocyte cell lines producing specific antibody. *Nature* **269**: 420-2.
- Stiegler G, Armbruster C, Vcelar B, Stoiber H, Kunert R, Michael NL, Jagodzinski LL, Ammann C, Jager W, Jacobson J, Vetter N, Katinger H, 2002, Antiviral activity of the neutralizing antibodies 2F5 and 2G12 in asymptomatic HIV-1-infected humans: a phase I evaluation. *AIDS* **16**: 2019-2025.
- Stogner SW, King JW, Black-Payne C, Bocchini J., 1993, Ribavirin and intravenous immune globulin therapy for measles pneumonia in HIV infection. *South. Med. J.* **86**: 1415-8.
- Sui J, Li W, Murakami A, Tamin A, Matthews LJ, Wong SK, Moore MJ, Tallarico AS, Olurinde M, Choe H, Anderson LJ, Bellini WJ, Farzan M, Marasco WA, 2004, Potent neutralization of severe acute respiratory syndrome (SARS) coronavirus by a human mAb to S1 protein that blocks receptor association. *Proc. Natl. Acad. Sci. USA* **101**: 2536-2541.
- ter Meulen J, Bakker AB, van den Brink EN, Weverling GJ, Martina BE, Haagmans BL, Kuiken T, de Kruif J, Preiser W, Spaan W, Gelderblom HR, Goudsmit J, Osterhaus AD, 2004, Human monoclonal antibody as prophylaxis for SARS coronavirus infection in ferrets. *Lancet* **363**: 2139-41.
- Thompson J, Pope T, Tung JS, Chan C, Hollis G, Mark G, Johnson KS, 1996, Affinity maturation of a high-affinity human monoclonal antibody against the third hypervariable loop of human immunodeficiency virus: use of phage display to improve affinity and broaden strain reactivity. *J. Mol. Biol.* **256**: 77-88.

- Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, Murphy BR, Rappuoli R, Lanzavecchia A., 2004, An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat. Med.* **10**: 871-5.
- van den Brink EN, ter Meulen J, Cox F, Jongeneelen MA, Thijsse A, Throsby M, Marissen WE, Rood PM, Bakker AB, Gelderblom HR, Martina BE, Osterhaus AD, Preiser W, Doerr HW, de Kruif J, Goudsmit J, 2005, Molecular and biological characterization of human monoclonal antibodies binding to the spike and nucleocapsid proteins of severe acute respiratory syndrome coronavirus. *J. Virol.* **79**: 1635-44.
- Wang CY, Sawyer LS, Murthy KK, Fang X, Walfield AM, Ye J, Wang JJ, Chen PD, Li ML, Salas MT, Shen M, Gauduin MC, Boyle RW, Koup RA, Montefiori DC, Mascola JR, Koff WC, Hanson CV, 1999, Postexposure immunoprophylaxis of primary isolates by an antibody to HIV receptor complex. *Proc. Natl. Acad. Sci. USA.* **96**: 10367-10372.
- Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar, MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM, 2003, Antibody neutralization and escape by HIV-1. *Nature* **422**: 307-312.
- Wolbank S, Kunert R, Stiegler G, Katinger H, 2003, Characterization of human class-switched polymeric (Immunoglobulin M [IgM] and IgA) anti-human immunodeficiency virus type 1 antibodies 2F5 and 2G12. *J. Virol.* **77**: 4095-4103.
- Wu H, Pfarr DS, Tang Y, An LL, Patel NK, Watkins JD, Huse WD, Kiener PA, Young JF. Ultra-potent antibodies against respiratory syncytial virus: effects of binding kinetics and binding valence on viral neutralization. *J. Mol. Biol.* **350**: 126-144.
- Xu W, Smith-Franklin BA, Li PL, Wood C, He J, Du Q, Bhat GJ, Kankasa C, Katinger H, Cavacini LA, Posner MR, Burton DR, Chou TC, Ruprecht RM, 2001, Potent neutralization of primary human immunodeficiency virus clade C isolates with a synergistic combination of human monoclonal antibodies raised against clade B. *J. Hum. Virol.* **4**: 55-61.
- Yang W-P, Green K, Pinz-Sweeny S, Briones AT, Buron DR, Barbas CF, 1995, CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV antibody into the picomolar range. *J. Mol. Biol.* **254**: 392-403.
- Zwick MB, Parren PW, Saphire EO, Church S, Wang M, Scott JK, Dawson PE, Wilson IA, Burton DR, 2003, Molecular features of the broadly neutralizing immunoglobulin G1 b12 required for recognition of human immunodeficiency virus type 1 gp120. *J. Virol.* **77**: 5863-5876.
- Zwick MB, Wang M, Poignard P, Stiegler G, Katinger H, Burton DR, Parren W, 2001, Neutralization synergy of human immunodeficiency virus type 1 primary isolates by cocktails of broadly neutralizing antibodies. *J. Virol.* **75**: 12198-12208.
- Zwick MB, Jensen R, Church S, Wang M, Stiegler G, Kunert R, Katinger H, Burton DR, 2005, Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. *J. Virol.* **79**: 1252-1261.