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Development of Human and Macaque Antibodies Using Antibody Phage Display for the Detection of Equine Encephalitis Viruses

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1. Introduction

Antibody phage display is an *in vitro* technology to generate recombinant antibodies. In particular for pathogens or toxins, antibody phage display is an alternative to hybridoma technology, since it circumvents the limitations of the immune system. Furthermore, phage display allows generation of human antibodies when either immunised patients are not available or immunisation is not ethically feasible.

Equine encephalitis viruses, like VEEV, WEEV and EEEV, belong to the Alphavirus group. Several species and subspecies of this family are pathogenic for man and are recognized as potential agents of biological warfare and terrorism. In this review, we describe the generation of human antibodies from naive antibody gene libraries and macaque antibodies from immune antibody gene libraries. Furthermore, we give an overview about phage display derived recombinant antibodies against equine encephalitis viruses for diagnostics and therapy.

2. Short introduction to encephalitis viruses

Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV) and Eastern equine encephalitis virus (EEEV) were first isolated in the 1930s and belong to the Alphavirus genus within the *Togaviridae* family (Giltner and Shahan, 1933; King, 1939; Kubes and Ríos, 1939; Meyer et al., 1931; Powers et al., 2001; Weaver et al., 2004). Mosquitoes are the biological vectors of theses viruses and equine species and man are periodically infected

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(Strauss and Strauss, 1994). In the past epidemics caused by these viruses occured in North, Central and South America. These viruses can spread to the human central nervous system (CNS) causing symptoms ranging from mild febrile reactions to encephalitis, often resulting in fatal and permanent neurologic damages. The severity of the illness depends on virus strain, age of patients as well as dose and route of infection (Calisher, 1994; Deresiewicz et al., 1997; Feemster, 1938; Hoke, 2005; Leitenberg, 2001; Letson et al., 1993; Rivas et al., 1997; Rozdilsky et al., 1968; Sanchez et al., 1984; Sellers and Maarouf, 1993; Sudia et al., 1975; Weaver et al., 1996). Moreover, alphaviruses can be produced in large quantities, are moderately easy to disseminate and are highly infectious as aerosols (Phillpotts, 2006; Reed et al., 2005). Therefore, VEEV, WEEV and EEEV are potential biological weapons (Hawley and Eitzen, 2001). All three encephalitis virus species are classified as Bioterrorism Agent Category B by the Center of Disease Control (CDC) (http://www.bt.cdc.gov/agent/agentlist-category.asp).
For rapid detection, diagnosis and treatment of VEEV, WEEV and EEEV antibodies are indispensable tools and potential therapeutics.

3. Antibody phage display

Production of polyclonal antibodies by immunisation of animals is a method established for more than a century. The first antibody serum was produced in horses and directed against diphtheria (von Behring and Kitasato, 1890). Hybridoma technology was the next milestone, allowing the production of monoclonal antibodies (Köhler and Milstein, 1975). Hybridoma technology has drawbacks like limited number of candidates, possible instability of aneuploid cell lines (Pauza et al., 1993), inability to provide antibodies against highly conserved antigens and most of all its limited application to generate human antibodies (Winter and Milstein, 1991). Hybridoma technology thus essentially allows isolation of murine antibodies which have a broad detection range and can be applied for diagnostic or research uses. However, their therapeutic applications are limited because repeated administration of murine antibodies can cause human anti-mouse antibody reaction (HAMA), in turn leading to a diminution of the antibody half-life and to severe side effects including anaphylactic side effects (Courtenay-Luck et al., 1986; Tjandra et al., 1990). These difficulties might be alleviated by engineering these antibodies to reduce their fraction of murine origin. Chimerised antibodies have murine variable regions, retaining binding capacity of the original murine antibody, expressed in fusion with human constant regions. But, these variable murine regions are still large enough to be a cause for adverse reactions so that the smaller hypervariable regions, in direct contact with the antigen, might be preferred as the only murine part retained in the therapeutic molecule. The corresponding engineering process is known as humanisation, which is however more difficult to implement than chimerisation. Another possibility is to utilise transgenic mice whose loci coding antibodies have been replaced by their human counterparts. With these mice, human antibodies can be generated by hybridoma technology but these transgenic animals are available in a very limited number of laboratories only (Fishwild et al., 1996; Jakobovits, 1995; Lonberg and Huszar, 1995; Nelson et al., 2010). For the isolation of antibodies directed against toxins, animals are immunised by non-toxic subunits or even selected domains from these subunits (Pelat et al., 2009a; Pelat et al., 2007; Scotcher et al., 2010; Winterroth et al., 2010).
A technology which circumvents the limitations of the immune system is antibody phage display. This technology is completely independent from any immune system by an *in vitro* selection process. The display method most commonly used today is based on the work of

George P. Smith (Smith, 1985) on filamentous phage, which infect *E. coli*. By this technology, genotype and phenotype of a polypeptide e.g. an antibody are linked by fusing the antibody-encoding DNA to the coat protein III gene (gIII) of the filamentous bacteriophage M13. The resulting antibody::pIII fusion protein is displayed on the surface of phage particles, thus allowing affinity isolation of the antibody and consequently the purification of its coding DNA present within the phage (Breitling et al., 1991; Clackson et al., 1991; McCafferty et al., 1990).

In fact only antibody fragments like single chain fragment variable (scFv) or fragment antigen binding (Fab) are used routinely for phage display (Hoet et al., 2005; Hust et al., 2010), due to limitations of the *E. coli* protein folding machinery which can express full-sized IgGs only in rare cases (Mazor et al., 2007; Simmons et al., 2002). ScFv fragments consist of the variable domains of the heavy and light chain (Fv fragment) connected by a peptide linker (Bird et al., 1988; Huston et al., 1988). Other used formats for phage display are single chain Fabs (scFab), human VH domains (dAbs) and the variable domains of camel heavy chains (VHHs) (Holt et al., 2003; Hust et al., 2007b; Muyldermans, 2001; Muyldermans et al., 2009). Another interesting „antibody" format for phage display are immunoglobulins of sharks (IgNARs), which are very resistant to denaturation and well suitable for diagnostics. (Flajnik and Dooley, 2009; Nuttall et al., 2001; Nuttall et al., 2004).

The phage-displayed libraries are built from immunised or non-immunised lymphocyte donors or from synthetic repertoires. Libraries using the naïve IgM repertoire - the primary immune response - of a donor or synthetic antibody sequences are summarised as „single-pot" or universal libraries. These libraries are designed to isolate antibody fragments binding to every possible antigen, at least in theory (Dübel et al., 2010; Winter et al., 1994). Different types of universal libraries are available. Naïve libraries are constructed from rearranged V genes from B cells (IgM) of non-immunised donors. Examples for this library type are the naive human Fab library constructed by de Haard et al. (de Haard et al., 1999) and the HAL scFv libraries (Hust et al., 2011). Semi-synthetic libraries are constructed from unrearranged V genes from pre B germline cells (Griffiths et al., 1994) or from an antibody framework (Pini et al., 1998) in which one or several complementarity determining regions (CDR), including always the CDR H3, are randomised. For the FAB310 antibody gene library, a combination of naive and synthetic repertoire was used. Here, light chains from autoimmune patients were combined with a Fd fragment containing synthetic CDR1 and CDR2 in the human VH3-23 framework and naive CDR3 regions, derived from autoimmune patients (Hoet et al., 2005). Fully synthetic libraries are made of human frameworks with randomised CDR cassettes (Knappik et al., 2000; Rothe et al., 2008). Universal libraries have a repertoire of 10^8 - 10^{11} independent clones as molecular repertoire for phage display selection procedures. The difficulty of naïve libraries is that antibodies isolated thereof have lower affinities as hybridoma derived antibodies. Affinity and stability of the antibodies selected by phage display can be increased by additional *in vitro* affinity maturation steps (Finlay et al., 2009; Kobayashi et al., 2010; Thie et al., 2011; Thie et al., 2009).

To date, antibody phage display was used for the generation of antibodies against a large panel of human pathogenic viruses by using either recombinant viral proteins or complete virus particles for panning. Naïve antibody gene libraries were used to generate antibodies against Sin nombre virus (Velappan et al., 2007), Dengue virus (Cabezas et al., 2008; Moreland et al., 2010), Hepatitis C virus (Songsivilai and Dharakul, 1998), Influenza virus (Lim et al., 2008) or VEEV (Kirsch et al., 2008).

Opposite to these naive libraries are "immune libraries" which are constructed from antibody V-genes isolated from IgG secreting plasma cells from blood or bone marrow of

immunised donors. V genes of these libraries contain hypermutations and are affinity matured. From these immune libraries, human antibodies usable for therapeutic purposes against infectious pathogens (Clackson et al., 1991; Pelat et al., 2007; Schütte et al., 2009), including viruses (Duggan et al., 2001; Hunt et al., 2010; Kang et al., 2006; Sun et al., 2009; Throsby et al., 2008) have been isolated. Generally, immune libraries have a size of 10^6-10^8 independent clones, but can not be built for each antigen of interest, because man cannot be immunized with all antigens for ethical and practical reasons. An alternative are macaque immune libraries. The antibody genes of non-human primates (NHP) are closely related to human antibody genes (Pelat et al., 2009b) and might easily be humanised or germline humanised. In this latter case, the macaque amino acid sequence of the antibody is first compared with the corresponding human germline sequence. Differing macaque amino acid sequences are localised and are replaced by their human germline counterparts (Pelat et al., 2008; Pelat and Thullier, 2009) with respect to their affinity. Human germline sequences belong to the immunological self and are generally perfectly tolerated. However, this approach can even be used to reduce the potential immunogenicity of human antibodies.

The *in vitro* isolation of antibody fragments from libraries is performed independently from their immune or naïve origin. It relies on the binding activity of these antibody fragments and is called „panning" (Fig. 1), referring to the gold digger's tool (Parmley and Smith, 1988). For this panning, the antigen (a protein or a complete organism like a virus) is immobilised to a solid surface, mainly plastic surfaces like polystyrene tubes (Hust et al., 2002) or 96 well microtitre plates (Hust et al., 2011) and incubated with antibody phage of the antibody gene library. During this incubation step, chemical (e.g. pH), physical (e.g. temperature) or biological (e.g. competitor) parameters can be controlled to select antibodies which are able to bind the antigen. Antibody phage particles which bind weakly to the antigen and the vast excess of non-binding antibody phage are removed by strigent washing. Specifically binding antibody phage are eluted (e.g. by trypsin or pH shift) and reamplified by infection of *E. coli*. Subsequently, the phagemid bearing *E. coli* are infected with a helperphage, mainly M13K07, to produce new antibody phage which can be used for further panning rounds until a significant enrichment of antigen specific phage is achieved. Usually two or three panning rounds are necessary to select specifically binding antibody fragments. After panning, soluble individual monoclonal antibody fragments or antibody phage are produced and specific antigen binding is analysed by ELISA to identify individual binders. Afterwards, these individual binders can be sequenced and further biochemically characterised (Hust et al., 2007a; Winter et al., 1994). This panning process can also be performed in a high-throughput manner (Buckler et al., 2008; Hallborn and Carlsson, 2002; Hust et al., 2011; Konthur et al., 2005). Because the gene sequence of the binder is available, the antibody – depending on the desired application – can be converted into different antibody formats (e.g. scFv-Fc fusion or IgG) and produced in different production hosts (Hust et al., 2011; Schirrmann et al., 2008). In summary, recombinant antibody fragments derived from phage display can be adapted to the final assay requirements.

4. Antibodies for diagnostics and therapy of encephalitis viruses generated by phage display

In the beginning of 2011, 29 therapeutic monoclonal antibodies were approved in the USA or EU. For further antibodies approval is pending (http://www.landesbioscience.com /journals/mabs/about). The most admitted therapeutic antibodies are for cancer and

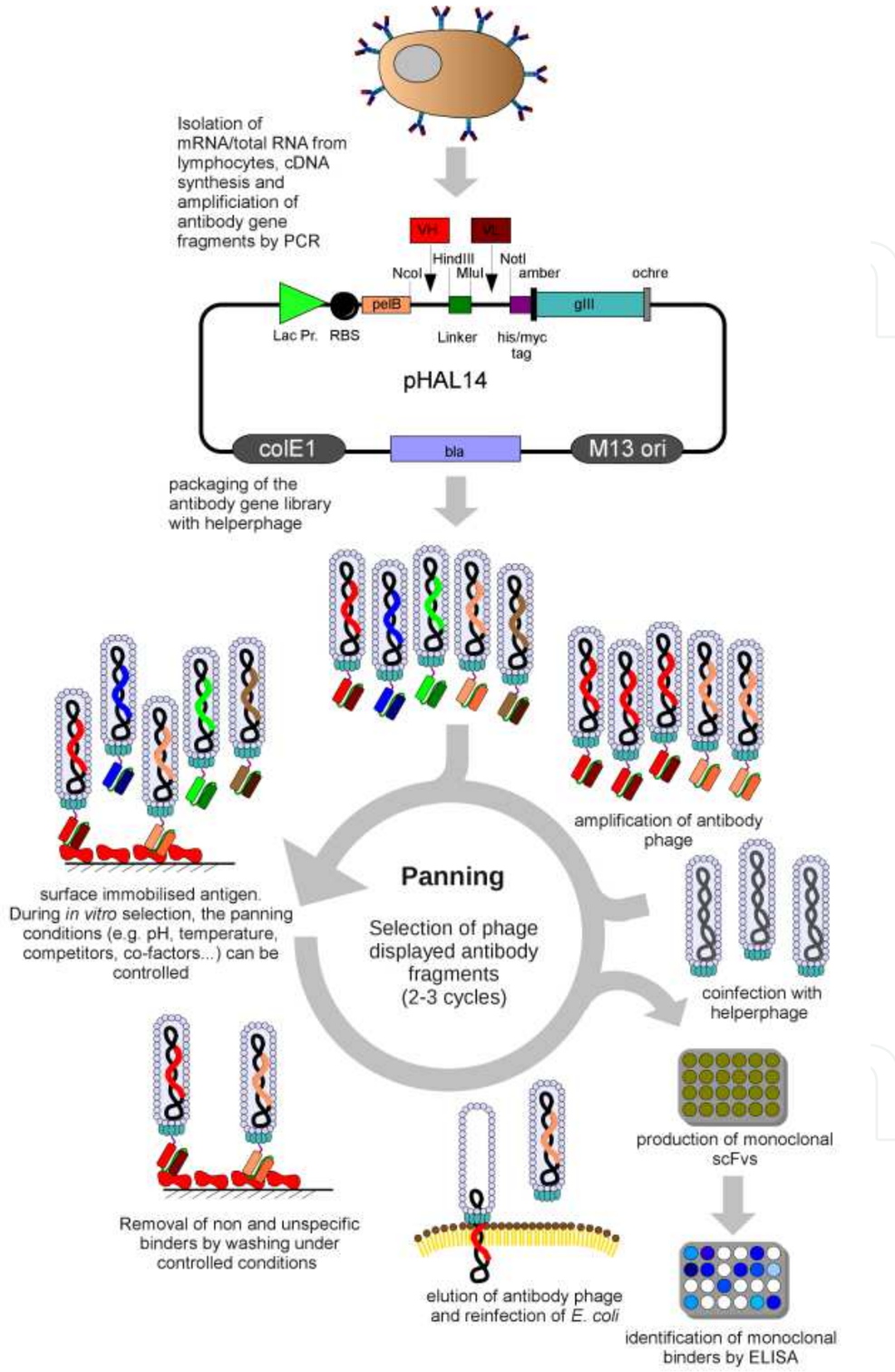

Fig. 1. Schematic overview about antibody gene library construction and the selection of binders (panning). This figure is a modified version of a former figure (Schirrmann and Hust, 2010).

autoimmune diseases (Dübel, 2007). Mechanisms of therapeutic antibodies are manifold and include neutralisation of substances e.g. toxins (Pelat et al., 2007) or cytokines like tumor necrosis factor (TNF) alpha (Alonso-Ruiz et al., 2008), blocking of receptors like epidermal growth factor receptor (EGFR) (Peeters et al., 2009), binding to cells and modulating the host immune system (Chatenoud and Bluestone, 2007), or combinations of these effects (Adams and Weiner, 2005). Interestingly, todate no recombinant antibodies are approved for the treatment of viral infections.

Rapid diagnosis of equine encephalitis is essential to allow an appropriate medical treatment, preventing viral replication. This may be allowed by point-of-care diagnosis tests based on antibodies or antibody fragments. After successful diagnosis, specific treatment might be considered and the use of recombinant (chimerized, humanized or human) neutralising antibodies is one choice. Also a prophylactic use of these recombinant antibodies might be considered for a time-limited protection, as human antibodies have a half-life of around 3 weeks in man (Eklund et al., 1982).

Since the administration of the original vaccine VEEV strain, TC83, developed in the sixties, had serious side effects to man (MCKINNEY et al., 1963), murine, macaque, humanised and human antibodies have been generated against VEEV by antibody phage display as an alternative in recent years. VEEV TC83 was used for example for the immunisation of mice from which the protective monoclonal antibody mAb 3B4C-4 was generated and later humanised (Hunt et al., 2006; Roehrig et al., 1982). The resulting antibody, mAb Hy4, turned out to be as protective as the original mouse antibody as demonstrated by virus challenge in Swiss Webster mice (Hunt et al., 2006). A further neutralising humanised antibody was developed by Hu et al (Hu et al., 2010).

The first anti-VEEV recombinant antibody fragments were derived from the murine IgG mAb 5B4D-6 by molecular cloning (Alvi et al., 1999). Murine scFvs aimed for diagnostic use were also isolated by phage display using immune libraries (Duggan et al., 2001), as phage display allows testing more candidates than hybridoma technology thus improving the search for selected specificities. Later, protective murine antibody fragments were generated using phage display. The broadly reacting scFv CUF37 was converted into the murine IgG2a format; this antibody was not neutralising *in vitro* but protective *in vivo* (O'Brien et al., 2009). The humanised version of this antibody (hu1A3B-7) protected mice in challenge experiments using the VEEV strain Trinidad Donkey (TrD) (Goodchild et al., 2011).

Regarding human antibody fragments, a panel of scFvs was selected for diagnostic assays from the naïve human libraries HAL4/7 using the antibody-displaying phage particles directly (Kirsch et al., 2008). Further human antibodies for therapeutic purposes were isolated using Fab (Fragment antigen binding) antibody phage display. The human immune library was constructed from sera of two VEEV strain TC-83 immunised US soldiers and two neutralising antibody fragments were identified. Hunt and coauthors suggested to use mAb F5 in combination with the earlier isolated humanised antibody mAb Hy4 for therapeutic purposes because they bind to different epitopes of the VEEV E2 glycoprotein (Hunt et al., 2010). The authors of the present review isolated a macaque scFv from an immune phage-displayed library directed against VEEV. Expressed as fusion with human constant regions, the resulting scFv-Fc protected mice against lethal challenge with Venezuelan equine encephalitis viruses (Rülker et al., in preparation).

To our knowledge no anti-WEEV antibody fragments derived from any kind of library is published. However, scFvs were generated from the murine IgGs 11D2 (Das et al., 2004; Xu

et al., 1999) and 10B5 E7E2 (Long et al., 2000) for diagnostic purposes. The authors of this review generated scFvs against WEEV by phage display using a macaque immune antibody gene library (Rülker et al. in preparation), both for diagnosis and therapeutic purposes.
Regarding EEEV, to our knowledge no recombinant human or murine antibodies have been published.
"At present there is no FDA-approved medical treatment for infection with these viruses." (Reichert et al., 2009). However, antibody phage display and other techniques have already delivered human or human-like antibodies as leads for the treatment or prophylaxis of equine encephalitis. Alternatively, the epitopes of these antibodies might also be candidate-vaccine.

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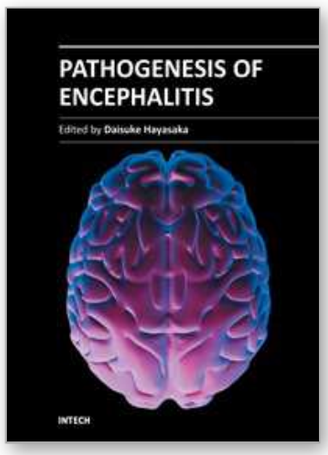

Pathogenesis of Encephalitis
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Many infectious agents, such as viruses, bacteria, and parasites, can cause inflammation of the central nervous system (CNS). Encephalitis is an inflammation of the brain parenchyma, which may result in a more advanced and serious disease meningoencephalitis. To establish accurate diagnosis and develop effective vaccines and drugs to overcome this disease, it is important to understand and elucidate the mechanism of its pathogenesis. This book, which is divided into four sections, provides comprehensive commentaries on encephalitis. The first section (6 chapters) covers diagnosis and clinical symptoms of encephalitis with some neurological disorders. The second section (5 chapters) reviews some virus infections with the outlines of inflammatory and chemokine responses. The third section (7 chapters) deals with the non-viral causative agents of encephalitis. The last section (4 chapters) discusses the experimental model of encephalitis. The different chapters of this book provide valuable and important information not only to the researchers, but also to the physician and health care workers.

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