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Research paper

A dimerized single-chain variable fragment system for the assessment of neutralizing activity of phage display-selected antibody fragments specific for cytomegalovirus

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ABSTRACT

Cytomegalovirus (CMV) causes severe sequelae in congenitally infected newborns and may cause life-threatening disease in immuno-deficient patients. Recent findings demonstrate the possibility to alleviate the disease by infusing intravenous immunoglobulin G (IgG) preparations, indicating that antibodies are an effective therapeutic option. Modern molecular methodologies, like phage display, allow for the development of specific antibodies targeting virtually any antigen, including those of CMV. However, such methodologies do not in general result in products that by themselves mediate biological activity. To facilitate a semi-high-throughput approach for functional screening in future efforts to develop efficacious antibodies against CMV, we have integrated two different approaches to circumvent potential bottlenecks in such efforts. Firstly, we explored an approach that permits easy transfer of antibody fragment encoding genes from commonly used phage display vectors into vectors for the production of divalent immunoglobulins. Secondly, we demonstrate that such proteins can be applied in a novel reporter-based neutralization assay to establish a proof-of-concept workflow for the generation of neutralizing antibodies against CMV. We validated our approach by showing that divalent antibodies raised against the antigenic domain (AD)-2 region of gB effectively neutralized three different CMV strains (AD169, Towne and TB40/E), whereas two antibodies against the AD-1 region of gB displayed minor neutralizing capabilities. In conclusion, the methods investigated in this proof-of-concept study enables for a semi-high-throughput workflow in the screening and investigation of biological active antibodies.

1. Introduction

More than half of the global human population is latently infected with human cytomegalovirus (CMV), a prototypical β-herpesvirus. The majority of CMV-infections are inapparent due to a functional immune system, which confines viral replication and provides protection against disease. However, immunodeficient individuals (e.g. AIDS or transplant patients) or infants that have been infected prior to birth are risk groups that suffer substantially from an infection since primary or recurrent infections may lead to life-threatening symptoms or severe disabilities. Considering the severe sequelae, for example sensorineural hearing loss or mental retardation in newborns or retinitis in immuno-compromised individuals, that CMV cause in these risk groups, there is a great need for a vaccine that induce protective immunity against CMV (Stratton et al., 2001). Importantly, in a recent study, Griffiths et al. (2011) were able to show reduced viremia and a shorter duration of therapy with antiviral agents following vaccination with a

Abbreviations: AD, antigenic domain;CMV, cytomegalovirus;gB, glycoprotein B;HSV, herpes simplex virus;scFv, single-chain variable fragment(s)

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recombinant viral glycoprotein. Unfortunately, recent infection-studies with the highly homologous rhesus CMV in macaques *in vivo* have challenged our hope for a truly efficacious protective vaccine (Hansen et al., 2010). Nevertheless, the ability of immuno-competent individuals to control the virus without signs of illness indicates that at least the viral disease can be prevented by an induced immune response. Indeed, several lines of evidence indicate that antibodies are important in this respect (Jonjic et al., 1994; Klenovsek et al., 2007; Nigro and Adler, 2010; Slavuljica et al., 2010) and consequently, passive immunization using highly effective CMV-specific hyperimmune globulin preparations, has shown to be effective for prevention of disease caused by CMV (Nigro et al., 2005). Today, novel molecular tools allow us to understand the nature of protective antibody responses and potential to develop effective defined therapeutic antibody preparations against CMV *in vitro*.

CMV-specific monoclonal antibodies have been developed using a variety of techniques involving hybridoma technology, cell sorting and molecular engineering approaches (Ohlin et al., 1993; Spaete et al., 1994; Axelsson et al., 2009; Macagno et al., 2010; Pötzsch et al., 2011). With the advent of single cell sorting and *in vitro* display technologies it is now possible to rapidly generate monoclonal antibody fragments against virtually any antigen. These systems also enables for high-throughput development of recombinant antibody fragments using automated systems for selection and screening (Hallborn and Carlsson, 2002; Turunen et al., 2009; Hust et al., 2011). However, these techniques often involve the development and production of monovalent antibody fragments, a format that is not always suitable for assessment of neutralizing activity against CMV (Lantto et al., 2002b; Barrios et al., 2007). The biological activity of the monovalent antibody format may be poor for instance due to their small size, lack of ability to cross-bind epitopes or faster off-rate reaction kinetics compared to an intact antibody. Additionally, such antibody fragments lack the immunoglobulin Fc region that contributes to effector functions that are vital for biological activity of some CMV-specific antibodies (Spaete et al., 1994).

The combination of phage display technology and high-throughput assay systems is an attractive set of components in a workflow for the generation of specific antibodies against CMV. A limitation in the process (Fig. 1) is to rapidly advance from initial hits, where the antibody fragments are monovalently displayed on phages, to assessment of the biological function of initially identified binders. To address these issues we have investigated tools that are applicable in large scale screening efforts to identify specificities against CMV with appropriate biological activity. The aim is to combine tools facilitating semi-high throughput divalent recombinant antibody production (Moutel et al., 2009) and a rapid screening system to assess biological activity by using a newly developed luciferase-based neutralization test (Reinhard et al., 2011). In this proof-of-concept study, we demonstrate that this approach allows for detection of neutralizing CMV-specific recombinant antibodies. We suggest that this methodology will contribute to future development of antibodies with desired biological function against CMV. It may also provide essential information about functional activity of clones induced in efforts, which despite the associated difficulties, are aimed at developing an effective vaccine against CMV.

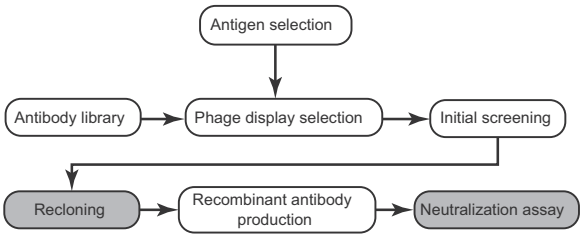

Fig. 1. Schematic illustration of major unit operations in a streamlined workflow to efficiently evaluate biological activity of binders specific for human CMV isolated in a phage display-based process. High-throughput methodology of phage display and subsequent screening processes have been described elsewhere (Turunen et al., 2009; Hust et al., 2011). The highlighted boxes represent the unit operations, described in detail in the Materials and methods section, exploited in this study as they hold capacity for increased process efficiency. A timeline for a semi-high throughput workflow of this kind is estimated to four–five weeks (provided that a suitable antibody library and antigens are available), based on repeated selection rounds using phage display and initial screening (altogether two to three weeks) and recombinant scFv-Fc production (one week). Cloning procedures and neutralization assays require 2 to 3 days each.

2. Materials and methods

2.1. Anti-CMV single-chain variable fragments

Recombinant single chain fragment variable (scFv) used in this study have previously been created from transcripts derived from human monoclonal hybridomas or selected from molecular libraries using phage display. The extensively characterized scFv are specific for different epitopes on CMV glycoprotein B: AD-1 (I2-1 and S2-8) (Barrios et al., 2007), AD-2 (AE11F, F3c10/2, D3-3(2)/11 and H3b/31) (Lantto et al., 2002a, 2002b, 2003). In addition, a less characterized binder (gB-17) (Axelsson et al., 2009) targeting an unidentified epitope on gB was also investigated. None of these scFv carry in their sequence motives for addition of N-linked carbohydrates.

2.2. Cloning of genes into vector for recombinant antibody production

Efficient generation of highly effective CMV-specific antibodies requires not only access to large numbers of recombinant binders for instance selected by phage display but also a semi-high throughput setup to analyze biological activity. This process also implies a need for binders in a divalent antibody format (Lantto et al., 2002b; Barrios et al., 2007). The basis for our production of divalent antibody fragments is a vector system that allows fast and easy transfer of large sets of genes encoding hit clones, identified in an initial screening process, for the production of recombinant antibodies in eukaryotic cells. A vector developed by Moutel et al. (2009) permits such direct transfer of genes encoding antibody fragments from vectors commonly used in phage display selections in one single cloning step. The system encodes a protein composed of one scFv fused to an Fc region of IgG (scFv-Fc) that homodimerize before secretion by eukaryotic cells.

CMV-specific scFv were cloned into the pFUSE-hIgG2-Fc2 plasmid (Moutel et al., 2009) in order to produce dimeric scFv-Fc fusion proteins. *Nco*I and *Not*I restriction sites are utilized to fuse genes encoding CMV-specific scFv with sequences encoding the Fc domain of human IgG2. The CMV-specific