Large-Scale Screening of Blood Donors for Exceptional Antibodies against Human Cytomegalovirus

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<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<td>BDCRB</td>
<td>2-Bromo-5,6-dichloro-1-β-d-ribofuranosyl benzimidazole</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>GCV</td>
<td>Ganciclovir</td>
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<td>GLuc</td>
<td>Gaussia luciferase</td>
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<td>HCMV</td>
<td>Human cytomegalovirus</td>
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<td>HIG</td>
<td>Hyperimmunoglobulin</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>HFFF-tet</td>
<td>Human fetal foreskin fibroblasts expressing tet repressor</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>NT50</td>
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I. Large-Scale Screening of Blood Donors for Exceptional Antibodies against Human Cytomegalovirus

1. CLINICAL IMPORTANCE & STUDY DESIGN

Clinical Importance

The human cytomegalovirus (HCMV) is a member of the herpesvirus family. Seroprevalence depends on socioeconomic status, age, gender and ethnicity, and consequently vary worldwide between 30 to 100%. (Bate, Dollard, and Cannon, 2010; Cannon, Schmid, and Hyde, 2010; Lachmann et al., 2018). HCMV, as all herpesviruses, persists in its human host and causes latent infection (Collins-McMillen et al., 2018; Goodrum, Caviness, and Zagallo, 2012; Reeves and Sinclair, 2013). Reactivation of virus leads to the presents of viral particles in body fluids, as blood, urine or breast milk (Arora et al., 2010; Barbosa et al., 2018; Cannon, Hyde, and Schmid, 2011; Hamprecht et al., 2003). HCMV infection of immunocompetent individuals is often not recognized since it causes only mild flu-like symptoms at the most. The picture is rather different in immunocompromised individuals, such as AIDS patients or transplant recipients, and also in unborn fetuses. Transplant patients can severely suffer from HCMV infection or reactivation. Solid organ and hematopoietic stem cell transplant recipients have a high risk for HCMV infection that can lead to graft-failure, graft versus host disease and also increased susceptibility for other infections (Kotton et al., 2013; Ljungman, 2014; Ljungman et al., 2014; Razonable, 2013). Congenital HCMV infection is a major cause of non-inherited disabilities in children, manifested in sensorineural hearing loss and neurodevelopmental sequela. Intrauterine transmission of HCMV to the unborn fetus occurs in 2/1000 to 20/1000 of live-births (Boppana, Ross, and Fowler, 2013; Britt, 2018; Dollard, Grosse, and Ross, 2007; Manicklal et al., 2013; Mussi-Pinhata et al., 2009) becoming more seriously with earlier gestational age. Even when children had no clinical findings at birth, 10-15% developed long-term sequela (Boppana et al., 2013). It is remarkably that although anti-HCMV antibodies were present in seropositive mothers, they do not provide solid protection from infection or intrauterine transmission in congenital HCMV infections (Britt, 2017). This finding indicates that the antiviral activity of the naturally elicited humoral immune response might not be sufficiently effective enough in some cases. For example, reinfection of seropositive pregnant women with a new HCMV strain could be associated with intrauterine transmission of the newly acquired virus (Boppana et al., 2001; Yamamoto et al., 2010).
There is an effective antiviral therapy available but unfortunately, its application is limited in some critical clinical situations. Chemical substances as ganciclovir, aciclovir or cidofovir target the viral polymerase but nephrotoxic and myelotoxic side effects make their use complicated in transplant patients (Chen et al., 2018; Meesing and Razonable, 2018) and unsuitable for treatment of pregnant women. The recently developed terminase inhibitor letermovir is more compatible, but its application can be also restricted by the development of resistance (Bowman, Melaragno, and Brennan, 2016; Chemaly et al., 2014; Goldner, Zimmermann, and Lischka, 2015; Marty et al., 2017). Active or passive immunization would be an alternative treatment strategy with good compatibility. A lot of different vaccine strategies (e.g. a subunit vaccine based on the viral glycoprotein gB, replication defective viruses, DNA-vaccines) were developed but all efforts to prevent HCMV infection by active immunization did not result in a licensed vaccine yet (Permar et al., 2018; Plotkin and Boppana, 2018). Passive immunization strategies with neutralizing monoclonal antibodies or HCMV-hyperimmunoglobulins (HIG) already showed partially promising results. In stem cell transplant patients it was reported that there were positive clinical effects on acute graft-versus-host disease and CMV disease by HIG treatment, but morbidity and mortality were only marginally improved (Ahn et al., 2018; Bass et al., 1993; van Gent, Metselaar, and Kwekkeboom, 2017). In contrast, it was recently reported that significant reduction of maternal-fetal virus transmission could be achieved by bi-weekly HIG administration (Kagan et al., 2018). Also the combination of two HCMV-neutralizing monoclonal antibodies could reduce the frequency of HCMV viremia in kidney-transplant patients (Ishida et al., 2017). Passive immunization is a promising treatment strategy that relies on the common humoral immune response to HCMV at the moment. However, a superior antibody quality that is more effective compared to the commonly elicited antibodies, would be desirable to gain more definite results. The usage of superior antibodies may result in a satisfying passive immunization strategy which could be reliably effective independently on the clinical situation. In the HIV research field, passive immunization strategies based on superior antibodies have reached remarkable progress. Exceptionally highly potent and broad “elite neutralizers” (Simek et al., 2009; Walker et al., 2011) were successfully used for treatment and prevention of HIV viremia and transmission (Caskey et al., 2017; Mendoza et al., 2018; Possas et al., 2018). In view of those promising achievements, the intention was to improve the quality of anti-HCMV antibody preparations by following a related strategy.

**Study Design**

To accomplish this intention, plasma samples from a large human population were investigated regarding their capability to inhibit HCMV infection. A screening of thousands of blood donor plasma samples was supposed to identify exceptionally potent...
and broad antibodies. Consequently, a systematic screening algorithm was designed. According to strict quality criteria, it combines all important aspects of HCMV transmission: the two modes of viral spreading, its broad cell tropism and strain-dependent differences.

There are two modes of viral spreading occurring that were considered in our algorithm. Recent clinical HCMV isolates are strictly cell-associated, hence transmission of viral particles occurs only from cell to cell (Frascaroli and Sinzger, 2014; Yamane, Furukawa, and Plotkin, 1983). Following to passage of viral isolates in fibroblast cell culture, several genes (e.g. most prominently RL13, UL128, UL130 and UL131) acquire mutations that lead to a growth advantage by release of free viral particles into the supernatant (Adler, 2006; Dargan et al., 2010; Sinzger et al., 1999; Stanton et al., 2010). The cell-free mode of viral spreading is more efficient in the cell culture but which transmission mode is more relevant in vivo is not clearly known. Cell culture adapted HCMV strains spread via both ways, cell-free and cell-associated, whereas recent clinical HCMV isolates are strictly cell-associated. Nevertheless, free viral particles are also present in the human body fluids e.g. blood, urine and breast milk (Arora et al., 2010; Hamprecht and Goelz, 2017; Hamprecht et al., 2003). Since it is not clearly known which mode is more important for viral dissemination in the human body, we had to consider both for clinical application. We attempted to identify human blood donors out of a large population whose plasma can inhibit cell-free and cell-associated transmission. Since laboratory strains usually spread via both transmission routes in parallel, we had to do separate approaches. On the one hand, a screening for elite neutralizers, and on the other hand, a screening for inhibitors of cell-associated spread were performed. In follow-up studies, we added further quality aspects to the selection process. Since it is known, that HCMV uses two different glycoprotein complexes for entry into fibroblasts or endothelial and epithelial cells (Vanarsdall and Johnson, 2012), successful inhibition of dissemination would include effective antibody activity against both entry routes. In parallel, we considered that there are variations of neutralizing efficacy due to the strain exposed. Since it is not predictable which strain is present in the patient, a competent antibody should inhibit a wide spectrum of HCMV strains to be broadly applicable. Summarizing, beside potent activity of immunoglobulins the screening algorithm includes broadly effectiveness (i) independently of the cell-type that is infected and (ii) independently of the HCMV strain that is present.
2. IDENTIFICATION OF ELITE NEUTRALIZERS

Aim

In the first place, we intended to find exceptionally effective antibodies against cell-free infection. The most potent neutralizing antibodies should be identified out of thousands of plasma samples from HCMV-seropositive blood donors. As prior selection criteria, we defined high neutralizing capacities for infection of both model cell types, endothelial cells and fibroblasts. The most potent plasma samples were then further assessed regarding broad effectiveness against several HCMV strains.

Approach

The first step towards an analysis of several thousands of plasma samples was the establishment of a reporter virus that facilitates a high-throughput screening. We decided to use the secreted Gaussia-luciferase as a reporter. The endotheliotropic HCMV strain TB40/E-BAC4 was chosen as genetic background for the reporter virus because it reflects the broad cell-tropism of HCMV (Sinzger et al., 2008). The Gaussia luciferase was ectopically inserted into TB40/E-BAC4 and is expressed with IE-kinetics. Luciferase activities in the supernatant of infected cell cultures correlated with applied viral doses. The publication “Generation of a Gaussia luciferase-expressing endotheliotropic cytomegalovirus for screening approaches and mutant analysis” (Falk et al., 2016) summarizes all characteristics and reports on its suitability for determination of neutralizing capacities as well as screening approaches in a high-throughput fashion.

Results & Discussion

In the publication “A two-step screening approach for the identification of blood donors with highly and broadly neutralizing capacities against human cytomegalovirus” (Falk et al., 2017), a first set of 1000 plasma samples from human blood donors was analyzed with regard to set up a screening algorithm for potent and broad neutralization. Since it was previously reported that there are cell-type dependent differences for HCMV neutralization (Cui et al., 2017; Klein et al., 1999; Wang et al., 2011), we determined the neutralizing capacities (indicated as half-maximal neutralization titers, NT50) for the two major model cell types for HCMV infection. NT50s for infection of endothelial cells were compared to those for fibroblast infection. We found in concordance with others (Gerna et al., 2008; Revello and Gerna, 2010; Wang et al., 2011) that plasma samples from seropositive individuals were in general more effective in neutralizing endothelial cell
infection as compared to infection of fibroblast. This is probably due to different entry mechanism for the two major cell types. HCMV entry is mediated by two glycoprotein complexes, the pentameric complex gH/gL/UL128-131 and the trimeric complex gH/gL/gO. The entry of free-viral particles into endothelial cells is dependent on the pentameric complex whereas the entry into fibroblasts is pentamer-independent (Hahn et al., 2004; Vanarsdall, Chase, and Johnson, 2011; Wang and Shenk, 2005) (Adler, 2006; Ryckman, Chase, and Johnson, 2008; Zhou et al., 2015). The finding that plasmas showed in general higher NT50s for endothelial cell infection indicated that entry into endothelial cells is more sensitive to antibody attack. We concluded that a screening for potent neutralization of fibroblast infection would include potent neutralization for endothelial cells. Consequently, we simplified our screening algorithm by narrowing the high-throughput screening down to fibroblasts.

In parallel, we considered that neutralizing efficacy can be dependent on the HCMV strain that is exposed. The genotypes of envelope glycoproteins differ between HCMV strains (Chou, 1992; Chou and Dennison, 1991; Rasmussen et al., 2002; Mattick et al., 2004; Pignatelli, Dal Monte, and Landini, 2001; Pignatelli et al., 2004). Since we do not know which genotypes are present in the patient, antibodies for passive immunization should be potently effective against several strains. For this reason, we analyzed the identified exceptionally potent plasma samples according to their neutralizing activity against seven HCMV laboratory strains that represent nearly all genotypes of the most prominent glycoproteins. The resulting two-step screening algorithm was finally applied to additional 9000 seropositive plasma samples from human blood donors.

The screening was published in “Identification of elite neutralizers with broad and potent neutralizing activity against human cytomegalovirus (HCMV) in a population of HCMV-seropositive blood donors” (Falk et al., 2018b). We identified the most potent 2.6 % of 9000 seropositive plasmas. A set of 80 potent plasmas was then further analyzed with regard to their broadness of neutralizing activity. First, the assumption that high neutralization of fibroblast infection would include efficiency against endothelial cell infection was reevaluated for this set of plasma samples. Their cell type-independent efficacy was approved by determining NT50s for both cell types. In a second step, we tested for strain-independent efficacy. For this question, we determined the neutralizing capacities against seven laboratory strains with an ELISA-based neutralization assay. Those plasma samples that could reduce the infection of all seven strains to 50 % when being 100-fold diluted (NT50 = 100), were designated as broadly neutralizing. The majority (71%) of the 80 potent plasmas tested were also broadly effective according to our criteria. Leading to the assumption that the majority of donors selected in the initial screen were also broadly effective against different HCMV strains. Summarizing, we succeeded in identifying exceptionally potent plasma samples from blood donors that were cell-type and strain independent effective (= elite neutralizers). It is possible that high potency is a result of
antibodies that specifically target one necessary antigen that is conserved among viral strains and dispensable for entry into both cell types e.g. the fusion mediating glycoprotein gB. Alternatively, a diverse mixture of antibodies targeting several steps in the viral entry process might be present and simply a high quantity, e.g. due to frequent reactivation, is the reason for their good efficacy. As we intended to further investigate the mode of action of the 80 identified elite neutralizers, we used an approach in which the inhibition of attachment can be distinguished from the inhibition of penetration. It turned out that the majority inhibited, as expected, the penetration of viral particles into the cell, but two elite neutralizing plasmas could reduce the number of initially attached particles. We concluded, that we not only selected for a high quantity of efficient antibodies in the polyclonal mixture but rather also distinct qualities. We further pursued this aspect by testing whether those plasma samples were actually superior to the conventional HCMV-hyperimmunoglobulin preparations (HIG). For this comparison, two HIG and three elite neutralizers were standardized to their IgG content and neutralizing capacities were determined. Remarkably, the elite neutralizers were actually outperforming both HIG preparations. This finding emphasizes the possibility that elite neutralizing antibody preparations could be more effective in the clinical situation compared to conventionally available HIGs.

**Study Outlook**

To further assess the question of in vivo efficacy, a clinical study is necessary. A collaboration with the blood bank of the German Red Cross enables the revisiting of those blood donors with elite neutralizing plasma. A donation of plasma or peripheral blood cells is hence available for further investigation. Since we could show that neutralizing capacities of elite blood donors remained stable over several years (Falk et al., 2018b), this facilitates a direct clinical application of fresh frozen plasma from elite donors. It is intended to investigate whether elite plasma can prevent CMV reactivation in stem cell transplant recipients. The first hint will give a pharmacokinetic study assessed at the University Medical Center Ulm. Seropositive hematopoietic stem cell transplant recipients have a high risk for HCMV reactivation and HCMV disease (Ljungman, Hakki, and Boeckh, 2011). They should be weekly treated with plasma from elite neutralizers before and after transplantation. We would like to observe, as a first study endpoint, an increase of neutralizing capacities or anti-HCMV IgG quantitiy in the blood of transplant recipients after transfusion. Another passive immunization strategy could rely on monoclonal antibodies from elite donors. Others had successfully cloned the variable gene pairs of immunoglobulin heavy and light chains from human B-cells (Corti and Lanzavecchia, 2014; Wardemann and Busse, 2017). We also intend to gain outstanding antibody specificities from memory B
cells of selected elite donors. The first step towards this intention is the separation of HCMV-specific B-cells from all (EBV-immortalized) B-cells of a donor. Since the screening and selection process of antigen-specific B-cells from the whole B-cell repertoire is a crucial step, we approached this for HCMV by an antibody-footprint assay, publicized in “Detection of antibody-secreting cells specific for the cytomegalovirus and herpes simplex virus surface antigens” (Alt et al., 2018). Since HCMV-specific B-cells were not available in our lab, HCMV specific model cell types were used instead. This purpose served hybridoma cells or HEK-293T cells that express an HCMV-specific mouse or human antibody, respectively. The assay relies on the immobilization of secreted antibodies from living B-cells whereas only HCMV-specific antibodies were detected. B-cells were sedimented and secrete their antibodies which were bound to the microplate by capture antibodies. After removal of B-cells, GFP-expressing reporter virus binds exclusively to HCMV-surface specific antibodies only at those positions where the respective HCMV-specific B-cells had previously sedimented. Positions where GFP-reporter virus has bound to HCMV-specific B-cells, were then indirectly detected by infection of seeded fibroblasts. Wells with GFP-expressing foci reflect those wells where HCMV-specific B-cells were present before. This assay is beneficial as only B-cell specificities for viral surface-antigen epitopes were selected. Additionally, it is very sensitive as it detects single specific B-cells within hundreds of unspecific B-cells. This makes it suitable for a high-throughput screening of a whole B-cell repertoire in which the specific B-cells were hidden within thousands of unspecific. Subsequently to the selection process, it is intended to isolate the immunoglobulin heavy and light chain variable gene pairs by single-cell reverse transcription and express the antibodies in 293 cells.

The project could allow for an assortment of several broadly effective monoclonal antibodies from selected elite donors that are not only directed against different glycoproteins but also target different modes of action. A combination of several monoclonal antibodies, in line with previous passive immunization attempts (Bar-On et al., 2018; Ishida et al., 2017), could avoid upcoming resistance, hereby. An antibody preparation with elite monoclonal antibodies could be another step towards an improved passive immunization strategy.
3. SCREENING FOR INHIBITORS OF CELL-ASSOCIATED SPREAD

Motivation & Aim

Beside elite neutralizers, we intended to find inhibitors of cell-associated HCMV spread. In vitro studies of laboratory HCMV strains had shown that treatment with HCMV-seropositive serum or HIG can only inhibit the spread of free viral particles whereas foci remain (Jacob et al., 2013; Sinzger et al., 2007; Gardner and Tortorella, 2016). Although there are neutralizing monoclonal antibodies available (Boeckh et al., 2001; Gardner et al., 2016; Ishida et al., 2015), there is no antibody preparation known that can inhibit cell-associated HCMV spread on fibroblasts. This fact could be the reason for marginal effectivity of hyperimmunoglobulins in vivo. Against herpes simplex virus (HSV), there is a humanized mouse antibody mab2c which is directed against glycoprotein B that inhibits cell-associated spread and is also in vivo effective for prevention of keratitis in the mouse (Krawczyk et al., 2013; Krawczyk et al., 2015). Considering this aspect, we hypothesized that HCMV cell-to-cell spread inhibiting antibodies exist as well and that we might find them by screening a large human population. Consequently, we investigated 8300 seropositive plasma samples from human blood donors regarding their capability to inhibit the cell-associated spread of HCMV. The publication “Large scale screening of HCMV-seropositive blood donors indicates that HCMV effectively escapes from antibodies by cell-associated spread” reports on our findings.

Approach

Usually used laboratory HCMV strains spread via a combination of cell-free and cell-associated transmission. However recent clinical HCMV isolates spread strictly cell associated when being cultured in vitro. In order to investigate only inhibition of cell-associated spread with minimal effort in a high-throughput fashion, a virus with conditionally regulated growth behavior was required. HCMV strain Merlin_pAL1502, received from the Stanton lab, serves this prerequisite as it grows strictly cell-associated in primary human cells while release of virus can also be achieved by culturing it in HFFF-tet cells (human fetal foreskin fibroblasts with tet repressor). The possibility to gain cell-free virus was important as it circumvents an elaborate co-culturing of clinical-isolate infected cells with test cell cultures. Merlin_pAL1502 was derived by inserting the reconstructed genome of the clinical-isolate Merlin into a bacterial artificial chromosome (BAC) (Stanton et al., 2010). The viral genes, i.a. RL13 and the UL128 locus (containing UL128, UL130 and UL131A) were identified as modulators of growth behavior as they usually rapidly mutate by passaging a recent clinical-isolate in cell culture (Bradley et al.,
This mutations thereby allow for cell-free viral spreading. In Merlin_pAL1502, RL13 and UL128 were reconstructed and tet operators were inserted upstream of the translation regulation codons. In primary human cells, RL13 and UL128 were expressed and Merlin grows cell-associated like a clinical-isolate. In HFFF-tet cells, expressing the tet repressor, both genes were repressed and viral particles are released. For investigation of cell-associated spread in a high-throughput fashion, a reporter virus based on HCMV strain Merlin_pAL1502 was cloned. A secreted luciferase (Gaussia luciferase, GLuc) was inserted into the genetic backbone of HCMV strain Merlin_pAL1502 (Falk et al., 2018a). The ectopically inserted GLuc gene is regulated by the immediate early promotor. GLuc activity in the supernatant of infected cells reports on the level of infection. With Merlin_pAL1502-GLuc, 8400 plasma samples were screened for inhibition of cell-associated spread in fibroblasts. As positive controls for inhibition of cell-associated spread, the chemical antivirals ganciclovir (GCV) and BDCRB (2-Bromo-5,6-dichloro-1-β-d-ribofuranosyl benzimidazole) were used.

Results & Discussion

None of the plasma samples reduced the GLuc activity to the level the antivirals did. Those plasma samples that showed the highest reduction of GLuc activity were reevaluated by immunofluorescence staining but focal growth was not strikingly reduced either. Although a large cohort of human blood donors was investigated, a plasma that inhibited cell associated spread on fibroblasts could not be identified. Due to the fact that we previously had identified the elite neutralizers within the same donor population and the consideration that the same antigens for neutralization should be involved in cell-associated spread as well, it was remarkably that not a single plasma with a clear effect on cell-associated spread could be observed. To exclude that there is a strain dependent effect of Merlin, the effect of elite neutralizers was reevaluated with recent clinical isolates. A co-culture of three recent clinical isolates and fibroblasts was treated with six elite neutralizers. Repeatedly, we could not observe a striking effect on focal growth. Even elite sera that inhibited infection of free viral particles highly effectively, had little if any effect on cell-associated spread in fibroblasts.

There are cell-type dependent difference regarding cell-free spread and we observed this phenomenon also for cell-associated spread in our assay. When we tested our elite neutralizers for inhibition of focal growth of recent clinical-isolates in endothelial cells, cell-associated spread was partially sensitive. In endothelial cells, foci size could be inhibited up to 50 %. Similar results were obtained when anti-HCMV antibodies were applied to reduce focal growth in endothelial cells (Jiang et al., 2008) and also in epithelial cells (Cui et al., 2017; Kauvar et al., 2015). Summarizing, there is a cell-type dependent difference in the degree of resistance against neutralizing sera. While cell-associated spread in fibroblasts could not be inhibited even using exceptionally potent neutralizing
sera, focal spread in endothelial cells could be partially reduced but never completely (Fig. 1). This finding could be explained by the different entry routes into different cell types. Considering that pentamer-dependent entry into endothelial and epithelial cells can be inhibited more potently as compared with fibroblast entry, it may be possible that also in the context of cell associated spread, the pentamer-dependent spread is inhibited but the pentamer-independent spread remains. This possibly explains the small plaques that remain in an endothelial cell culture after treatment with neutralizing serum or antibodies. The fact that we could not identify a cell-to-cell spread inhibiting plasma within thousands of seropositive individuals clearly reveals that such a quality is extremely rare or does not exist at all. Nevertheless, it does not exclude that certain antibody qualities that could block cell-associated spread, are hidden in the polyclonal mixture and the investigation of B-cells from elite neutralizing monoclonal antibodies could reveal monoclonal antibodies that are effective when applied more concentrated as it is in human serum.

Figure 1: Screening algorithm for exceptional antibodies against HCMV. 9000 plasma samples from blood donors were screened for exceptional potent neutralization of cell-free viral particles on fibroblasts (= long-shaped cells). Exceptionally potent plasmas were then tested for broadness; which is cell-type independent (in fibroblasts and endothelial cells (= round-shaped cells)) and strain independent effectivity. “Elite neutralizers” were those plasma samples that were potently and broadly effective (extrapolation: ~1.8% of all plasmas). A part of the same donor repertoire was screened for inhibition of
cell-associated spread of HCMV in a different experimental set-up. Plasma samples showed no remarkably inhibition of focal growth. To reevaluate this finding, elite neutralizers were tested for inhibition of focal growth in different cell-types and different HCMV strains (cell-associated clinical isolates). For focal growth in fibroblasts, there was no striking effect detectable whereas focal growth in endothelial cells was partially inhibited but never complete.

Since the mechanism behind viral transfer from cell-to-cell is not known yet, failure of antibodies in inhibition of cell-associated spread could be due to several possible explanations. The antibody quantity could be insufficient for neutralizing the high number of viral particles that were transferred between cells. When we addressed this issue on the viral particle level, we did not find a reduction of transferred viral particles with antibody treatment (Falk et al., 2018a). Alternatively, the size of antibody molecules might not allow access to the areas between cells. A recent study from the Sinzger lab emphasizes this hypothesis as it has shown that peptide derivatives of PDGFRα, which is the cellular fibroblast receptor for HCMV entry, can inhibit cell-associated spread by inhibition of penetration (personal communication, 43rd Annual International Herpesvirus Workshop 2018, abstract no. 1.27)
4. SUMMARY

HCMV is still a clinical problem with serious consequences. It is the leading cause of disabilities in children due to congenital infection and also the reason for morbidity and mortality in immunocompromised patients. To improve existing passive immunization strategies, we investigated the human antibody response against HCMV regarding exceptionally effective antibodies that cover the main aspects of HCMV dissemination. For this purpose, a systematic screening algorithm was designed that was used to screen plasma samples from a large blood donor population. Since HCMV dissemination can occur via the cell-free and the cell-associated mode, both spread routes were addressed separately in two different approaches.

Human antibodies that are exceptionally effective in neutralizing free viral particles were identified by a two-step high-throughput screening. As a screening tool, an endotheliotropic HCMV strain with a secreted luciferase was generated. It facilitates simple and rapid detection of infection levels. A neutralization assay based on this reporter virus was used to investigate the neutralizing activity for infection of the two model cell-types for HCMV. In a first set of 1000 plasmas, the neutralizing capacities against fibroblast infection were compared to that in endothelial cells. We observed that plasma is in general more effective in neutralizing endothelial cell infection. Assuming that good neutralization in fibroblasts would include good neutralization of endothelial cell infection as well, the high-throughput screening was performed on fibroblasts. Consequently, the next 9000 plasma samples were tested on fibroblasts at a suitable dilution that would separate the plasmas with common neutralization from those with exceptionally potent neutralization. According to our cut-off, 2.6% of plasma samples were potently neutralizing. 80 of those potent plasma samples were then further investigated in a second screening step according to their efficacy against different HCMV strains. Plasmas that can be 100-fold diluted and still neutralized 50% infection (NT50 = 100) of all seven strains, were regarded as broad and potent. The screening algorithm identified “elite” neutralizing plasma samples that are exceptionally potently neutralizing with cell-type and strain-independent efficacy. Furthermore, it was shown that the elite plasmas are superior to conventional hyperimmunoglobulin preparations, hence potentially improving the passive immunization strategies against HCMV. The fact plasma samples with elite neutralizing capability can be traced back to the corresponding blood donors who can be revisited, could facilitate the usage of their plasma for direct application or the generation of monoclonal antibodies from their B-cells. The finding that neutralizing capacities of human blood donors against HCMV were stable over several years greatly facilitates this purpose.

Besides spreading via free viral particles, HCMV also spreads via a cell-associated mode.
Recent HCMV clinical isolates, are restricted to cell-associated growth whereas they lose this ability by passaging in cell culture. Since both transmission routes might play an important role in vivo, we investigated human plasma also regarding this transmission route. A suitable reporter virus was generated that grows strictly cell-associated like a clinical isolate. It facilitated the screening of 8400 plasma samples according to their inhibiting effect on HCMV cell-associated spread. Remarkably, this screening did not reveal a plasma that strikingly inhibited focal growth in fibroblasts. As we wanted to assure no false-negative results due to our test system, a second validation of this result was done. By usage of recent clinical isolates instead of the laboratory model strains for cell-associated spread, we excluded strain specific effects. Since we assume that the same glycoproteins that are involved in entry of free viral particles, also mediate cell-associated spread, we rechecked this issue with the previously identified elite neutralizing plasmas. But even the elite neutralizers did not inhibit cell-associated focal growth of clinical isolates on fibroblasts. In contrast, focal spread of clinical isolates between endothelial cells, can be partially inhibited by elite neutralizers while small foci remain. This reveals that there is a cell type dependent difference in inhibition not only regarding entry of free viral particles but also regarding cell-to-cell transmission of viral particles.

This study was designed to find individuals with exceptionally potent activity against HCMV. While we found that a quality of plasma with effective cell-to-cell spread inhibiting activity is nonexistent or extremely rare, we succeeded in identifying individuals with exceptional neutralizing activity against cell-free dissemination. This analysis could be the basis towards an improved passive immunization strategy against HCMV, relying on elite neutralizing antibodies that were potently and broadly effective and superior to conventional immunoglobulins.
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III. Publications
1. Generation of a Gaussia Luciferase-expressing Endotheliotropic Cytomegalovirus for Screening Approaches and Mutant Analysis

Jessica J. Falk, Kerstin Laib Sampaio, Cora Stegmann, Diana Lieber, Barbara Kropff, Michael Mach and Christian Sinzger

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Jessica Falk has cloned the reporter virus. The experiments „hybridoma-screening“ and „mutant analysis“ were done by Kerstin Laib Sampaio and Cora Stegmann, respectively. All other experiments were performed by Jessica Falk. Major parts of the first manuscript draft were written by Jessica Falk and all corresponding visualization. Reviewing and editing of the manuscript were done together with Christian Sinzger.
Generation of a Gaussia luciferase-expressing endotheliotropic cytomegalovirus for screening approaches and mutant analyses

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Abstract

For many questions in human cytomegalovirus (HCMV) research, assays are desired that allow robust and fast quantification of infection efficiencies under high-throughput conditions. The secreted Gaussia luciferase has been demonstrated as a suitable reporter in the context of a fibroblast-adapted HCMV strain, which however is greatly restricted in the number of cell types to which it can be applied. We inserted the Gaussia luciferase expression cassette into the BAC-cloned virus strain TB40-BAC4, which displays the natural broad cell tropism of HCMV and hence allows application to screening approaches in a variety of cell types including fibroblasts, epithelial, and endothelial cells. Here, we applied the reporter virus TB40-BAC4-IE-GLuc to identify mouse hybridoma clones that preferentially neutralize infection of endothelial cells. In addition, the Gaussia luciferase is secreted into culture supernatants from infected cells it allows kinetic analyses in living cultures. This can speed up and facilitate phenotypic characterization of BAC-cloned mutants. For example, we analyzed a UL74 stop-mutant of TB40-BAC4-IE-GLuc immediately after reconstitution in transfected cultures and found the increase of luciferase delayed and reduced as compared to wild type. Phenotypic monitoring directly in transfected cultures can minimize the risk of compensating mutations that might occur with extended passaging.

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1. Introduction

Human cytomegalovirus (HCMV) is a herpesvirus that can cause severe health problems particularly in the context of immature or compromised immune responses, e.g. when the virus is transmitted to the fetus during pregnancy or when transplant recipients or AIDS patients are infected. Antiviral chemotherapy is available and can repress viral replication in patients. However, all currently approved drugs have significant adverse effects, and therefore the development of new antiviral approaches is demanded (Ariza-Heredia et al., 2014; Gwee et al., 2014). In that context, reporter viruses can greatly facilitate high-throughput screening approaches as they allow fast and straightforward quantification of viral replication.

For example, HCMV strains expressing the firefly luciferase have been utilized to replace laborious immunofluorescence procedures with luciferase detection in virus neutralization assays (Potzsch et al., 2011; Scrivano et al., 2011; Wiegers et al., 2015). A disadvantage of the firefly luciferase is that it is retained inside the cell and therefore cells have to be lysed before the luciferase activity can be determined. In contrast, the Gaussia luciferase (GLuc) is naturally secreted from the producing cells into the extracellular fluid and hence its activity can be determined directly in culture supernatants without a need to kill the cells (Michelini et al., 2009), thus enabling repeated sampling over a period of time. A GLuc-expressing derivative of the laboratory strain AD169 has been successfully applied for analyses of mutations conferring drug resistance (Drouot et al., 2013). However, as this HCMV strain is restricted in its cell tropism due to a disruption in the viral UL131A gene (Adler et al., 2006; Wang and Shenk, 2005), it cannot be used for applications in endothelial or epithelial cells.

The TB40-BAC4 strain combines the effective replication of a laboratory strain with the broad cell tropism of recent HCMV isolates (Frascaroli and Sinzger, 2014; Sinzger et al., 2008). We have now inserted a GLuc expression cassette under the control of the CMV promoter (Bošhart et al., 1985; Thomsen et al., 1984) into the genome of this strain to generate a reporter virus with broad cell tropism that starts luciferase expression immediately after

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infection. We demonstrate that this virus can be used for screening approaches in endothelial and epithelial cell cultures. Detailed protocols are provided (i) for purification of virus-containing cell culture supernatants from contaminating luciferase secreted by producer cultures and (ii) their application in neutralization assays.

Another application of such a reporter virus is the phenotypic monitoring of mutant viruses. Cloning of HCMV genomes into bacterial artificial chromosomes (BACs) in combination with advanced genetic engineering techniques has greatly accelerated the targeted mutagenesis of HCMV genomes (Borst et al., 2004; Tischer et al., 2010). In contrast, the phenotypic characterization of such mutants is often very time-consuming. Here we show, how the GLuc reporter virus can be applied for fast and reliable quantification of viral growth immediately after reconstitution of recombinant virus from the mutant BAC clone.

2. Materials and methods

2.1. Cell culture

HEC-LTT is a conditionally immortalized cell line derived from primary human umbilical vein endothelial cells (HUVECs) (May et al., 2010). It has been shown that the HEC-LTT cells are useful to study endothelial cell infection by HCMV in vitro (Lieber et al., 2015). HEC-LTTs and HUVECs were cultured in endothelial growth medium (EGM BulletKit, Lonza). HEC-LTT medium was supplemented with 2 ml/g doxycycline until the day prior to the experiment. Human renal epithelial cells (HREs) were cultured in epithelial growth medium (REGM BulletKit, Lonza). Cell culture vessels for endothelial and epithelial cells were coated with 0.1% gelatin (Sigma-Aldrich). To ensure functional infectivity during experiments, EGM was removed from the cultures and HCMV infection was performed in minimum essential media (MEM) supplemented with GlutaMAX (Life Technologies), 5% fetal bovine serum and 100 ml/ml gentamicin. Human foreskin fibroblasts (HFFs) were cultured in MEM supplemented with GlutaMAX (Life Technologies), 5% fetal bovine serum, 0.5 ng/ml basic fibroblast growth factor (bFGF, Life Technologies) and 100 mg/ml gentamicin. During experiments, bFGF was omitted from HFF-medium (denoted as MEMS).

2.2. Generation of a GLuc-expressing reporter virus

2.2.1. Insertion of a GLuc expression cassette into the BAC-cloned virus strain TB40-BAC4

The reporter virus TB40-BAC4-IE-GLuc was constructed in the background of the bacterial artificial chromosome (BAC)-cloned strain TB40-BAC4 that retains a natural broad cell tropism and grows to high titers (Sinzer et al., 2008). To generate a transfer plasmid, the GLuc expression cassette was excised from plasmid pCMV-Gaussia Luc (ThermoFisher) with enzymes SpeI and SpII and cloned into SpII and AvrII sites of plasmid pDrive (Qiagen) resulting in plasmid pDrive-(IE-GLuc-PolyA). A fragment containing the kanamycin resistance gene and an I-SceI restriction site was amplified from plasmid pEPKan-S (Tischer et al., 2010) with primers 5’-ATATAGTCGACATCTTACGATCTCTGGTCTCAAAGCTTGGACCCATGTAGAGTTAATCAGGCCCAAGTAAATCAGTTGAGATTT-3’ and 5’-AAATGCTTTCCAGCGCTTGGATCCTACACCACTTAACC-3’, thereby adding a short duplication of GLuc sequences and Sall restriction sites. The PCR fragment was inserted into a single Sall site within the GLuc gene of plasmid pDrive-(IE-GLuc-PolyA), finally resulting in the universal transfer construct pDrive-(IE-GLuc-PolyA)-Kan. The (IE-GLuc-PolyA)-Kan fragment was amplified from this construct with primers: 5’-CAGTGGGAGGAGGCACCAGGAGACGATCAATCCTGAGATTT-3’ and 5’-GACATGTTAACCAGGGCTTCGTAGGATTAGAATATCAATTACG-3’ and electroporated into recombination-activated E.coli strain G51783 (Tischer et al., 2010) harboring TB40-BAC4. Following kanamycin selection, the marker cassette was removed from the BAC by an intrabacterial I-SceI digest and a Red-mediated recombination step. The integrity of the recombinant TB40-BAC4-IE-GLuc was verified by (i) restriction digestion using EcoRI to verify the pattern of restriction fragments and by (ii) sequence analysis of the recombinated regions by PCR with primers: 5’-TTTTGCGAGCTTTAGTG-3’ and 5’-TCCACATGTACGCGCTTAGC-3’. The sequence analysis revealed an N150D amino acid substitution within the luciferase coding sequence, which did not disable expression of a functional luciferase.

2.2.2. Purification of virus-containing cell culture supernatants from contaminating luciferase secreted by producer cultures

Crude virus stocks were obtained by collecting supernatants from infected HFF cultures at 5–7 days post infection (d.p.i.). These crude stocks contained contaminating luciferase secreted by producer cultures, which might cause disturbing background signals in subsequent assays. The supernatants were therefore centrifuged at 3220g for 10 min to remove cell debris. Viral particles were subsequently pelleted by ultracentrifugation for 70 min at 100,000g at 10 °C. The virus pellet was immediately resuspended in MEMS and subjected to a second round of centrifugation. Afterwards, the pellet was resuspended in a small volume of MEMS and stored in aliquots at −80 °C. This treatment was meant to preserve the biological activity of virions while removing soluble components, i.e. GLuc secreted from the producer cells. Virus stocks from which contaminating luciferase has been removed by ultracentrifugation are designated “luciferase-depleted” in the following text.

2.2.3. Measurement of GLuc activity

The measurement was performed by a microplate reader with a built-in injection system (Chameleon, TECAN). A 20 μl aliquot of each cell culture supernatant was transferred to a 96-well luminescence reader plate (LUMITRAC, Greiner Bio-One). The substrate coelenterazine (PKJ GmbH) was diluted to 0.2 μg/ml in phosphate buffered saline (PBS) supplemented with 5 mmol/l NaCl, and 50 μl of this working solution were automatically injected into each well immediately before the measurement. The light signal emitted during the luciferase reaction was measured with a total delay of 2.2 s and a counting time of 2 s. In case of saturation of GLuc activity signals, the GLuc-containing supernatants were diluted, remeasured and the relative light units (RLU) were subsequently multiplied by the respective dilution factor.

2.2.4. Expression kinetics of GLuc

HFFs were seeded on gelatin-coated 96-well plates at a density of 1.5 × 10^4 cells per well. Cells were infected with two separate luciferase-depleted TB40-BAC4-IE-GLuc preparations, each in a two-fold dilution series for 1 h at 37 °C. The virus was removed and the cultures were washed twice with MEMS. At 2, 8, 16 and 24 h.p.i., 13% (20 μl) of supernatants were removed and replaced by fresh medium. The samples were stored at 4 °C for a maximum of 24 h. The GLuc activity of the samples was measured at each time point and each virus concentration. If GLuc activities were above the linear range of the reader, samples were diluted, read again, and “relative light units” of the original samples were calculated by multiplication of the result with the dilution factor. In parallel, the
multiplicity of infection of both virus preparations was determined by a serial dilution assay as described below.

2.3. Phenotypic characterization of viruses

2.3.1. Detection of viral antigen

Cells were fixed with 80% acetone and incubated with primary mouse antibody E13 (Argene Biosoft) to detect immediate early (IE) antigens 1 and 2 (pUL122/123) (Mazeron et al. 1992). As a secondary antibody, Cy3-conjugated goat polyclonal anti-mouse Fab(ab)2 antibody (Jackson ImmunoResearch) was used. The nuclei of the cells were counterstained with DAPI (Sigma-Aldrich). Visualization was done by fluorescence microscopy with an Axio Observer D1 microscope (Zeiss).

2.3.2. Determination of virus titers by serial dilution

1.5 x 10^4 HFFs were seeded on gelatin-coated 96-well plates. On the following day, the cell cultures were inoculated with infectious supernatants at two-fold serial dilutions. At 1 d.p.i., the cultures were subjected to detection of viral IE antigens as described above. Two images per dilution step were taken, and the total number of nuclei (DAPI) or the number of IE antigen-positive nuclei (Cy3), respectively, were automatically counted. The infection rate was determined as the ratio of IE antigen-positive nuclei divided by the number of DAPI-positive nuclei viral titers were calculated as infectious units/ml

2.3.3. Single step growth curve

HFFs were seeded in gelatin-coated 6-well plates at a density of 2.5 x 10^3 cells per well. The next day, plates were infected with TB40-BAC4 or TB40-BAC4-IE-GLuc at a multiplicity of infection (MOI) of 3. Two hours post infection, virus was removed and the cultures were washed four times with MEM5. The following days, aliquots of the infectious supernatants were collected daily and replaced by equal volumes of fresh medium. The supernatants were centrifuged at 3220g for 10 min to remove cell debris and stored at -80 °C. The viral titer of each supernatant was determined by serial dilution assay as described above, and the results were plotted against the time after infection (Fig. 1B).

2.3.4. Cell tropism assay

HFFs, HUVECs or HREs were seeded in gelatin-coated 96-well plates with cell type-specific medium at a density of 1.5 x 10^4 cells per well. The following day, the medium was removed from the cells, replaced by MEM5 and preincubated for 30 min at 37 °C. Each cell type was infected with TB40-BAC4 or TB40-BAC4-IE-GLuc at comparable infection multiplicities. Following an incubation of 2.5 h at 37 °C, the infectious supernatants were replaced by the appropriate medium for each cell type. The next day, IE antigen-positive nuclei were detected by indirect immunofluorescence as described above.

2.4. GLuc-based neutralization assay

HFFs and HEC-LTTs were seeded in gelatin-coated 96-well plates at a density of 1.25 x 10^4 cells per well. Serum from an HCMV-immunized mouse (details will be published separately) was serially diluted in MEM5 and mixed with equal volumes of a luciferase-depleted preparation of TB40-BAC4-IE-GLuc at a concentration resulting in a final MOI of 1 in HFFs. The mixture was incubated for 2 h at 37 °C. Following a 30 min preincubation step of the cell cultures in MEM5, the medium was replaced by the virus-antibody mixture and incubated for 2 h at 37 °C. The virus-antibody solution was removed and replaced with the appropriate medium for each cell type. At 24 h.p.i., 20 μl of each supernatant was collected and luciferase activity was measured. The data was normalized to the sample showing the highest luciferase activity and subtracted from 1 to obtain the neutralization rates. The neutralization rates were plotted against the logarithm of the antibody concentration, given as fractions of undiluted antibody solution (e.g. log -3 representing a 1/1000 dilution of the serum or the hybridoma supernatant). The effective concentration of antibody solution that leads to 50% reduction of luciferase activity (50% neutralization concentration, NC50) was determined by fitting of
a sigmoidal dose response curve to the neutralization rates (Systat Software, Inc. SigmaPlot for Windows, version 11.0).

2.5. Gluc-based screening of hybridoma cultures

A hybridoma cell line was generated by fusing HCMV-immunized mouse B-cells with the myeloma cell line Sp2.0 according to standard polyethylene glycol (PEG) fusion procedures. Fused cells were distributed into twenty 96-well plates in HAT selection medium (RPMI supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine, 25 μM 2-Mercaptoethanol, 100 μM IL-6, 100 μM hypoxanthine, 4 μM aminopterin and 16 μM thymidine). Ten days post fusion, 100 μl hybridoma supernatant from each well was mixed with 10 μl of a luciferase-depleted TB40-BAC4-IE-GLuc preparation (5 × 10^5 IU/ml) and incubated at 37 °C for 2 h. As negative or positive control, two wells on each plate were prepared with HAT selection medium or hybridoma supernatant of a clone producing anti-gH monoclonal antibody 14-4b (Simpson et al., 1993), respectively. HFFs and HEC-LTts, seeded in gelatin-coated 96-well plates at a density of 1.25 × 10^4 cells/well, were preincubated with MEM5 for 30 min. The virus/hybridoma supernatant mixtures were diluted 2-fold with MEM5 and subsequently used to infect HFFs and HEC-LTts in parallel. After 2 h at 37 °C, the mixture was exchanged against the respective medium and cells were incubated at 37 °C for 18 h. From each well, 20 μl of supernatant was subjected to measurement of luciferase activity as described above.

2.6. Gluc-based analysis of HCMV mutants during reconstitution

A TB40-BAC4-IE-GLuc-UL74stop virus was generated by introducing two stop codons within the open reading frame of pUL74 at the amino acid positions 7 and 12. Mutagenesis was performed according to the protocol of Tischer et al. as described above using primers 5′-gtgcatcggcttctctctcgccgctggtcctcagctgagaagaagagtttctgatatcatactatatggcagaatataaat-3′ and 5′-tagaaatatagatgattctggtgataaataataaaaataataggaagatctgcttcgttgc tttttttttgtcagcactactataactataactataactataactataactataactataactata-3′ to amplify the marker cassette. In order to reconstitute and characterize the mutant virus, BAC-DNAs were isolated and transfected into HFFs in duplicates as described above. At 2 days post transfection (p.t.), duplicate cultures were merged and cells were seeded into 25 cm^2 flasks. At 6 days p.t., cells were detached and distributed into two 25 cm^2 flasks. Every third day thereafter, the cells were detached and reseeded into the same culture flasks. Aliquots of the supernatants were collected 24 h after each reseeding, stored at −20 °C and measurement of the luciferase activity was performed as described above. In order to verify the results, virus growth was also monitored as the increase of the percentage of IE positive cells. For this, with every reseeding 1.5 × 10^5 cells were seeded in triplicates onto 96-well plates and fixed with 80% acetone after 4 h attachment at 37 °C. Infection rates were determined by indirect immunofluorescence analysis as described above. The experiments were terminated when the cytopathic effect was visible in approximately 100% of the culture.

3. Results

3.1. Generation and characterization of the reporter virus

To generate a derivative of HCMV TB40-BAC4 that releases an easily detectable marker reflecting the initiation of viral gene expression, we aimed at expressing GLuc under the control of the viral major IE promoter/enhancer (Fig. 1A). For insertion of the GLuc reporter cassette into the viral genome we used a replacement strategy, because the genome size of HCMV appears to be limited and further addition of genetic material might impair the replication efficiency (Yu et al., 2002). To limit the increase in genome size, we replaced 1111 bp of the BAC cassette, containing the guanine phosphoribosyltransferase (gpt) gene, with a GLuc reporter cassette that is 1331 bp in size. The gpt gene had been used as a selection marker during the original cloning of the HCMV-BAC (Borst et al., 2004; Sinzger et al., 2008) but was no longer needed once the BAC clone was established.

Briefly, we generated the transfer plasmid pDrive-IE-GLuc/Kan-PolyA, from which an IE-GLuc/Kan-PolyA fragment was PCR-amplified, and we inserted the fragment into TB40-BAC4 in place of the gpt gene by “en passant mutagenesis” (Tischer et al., 2010). Subsequent removal of the kanamycin resistance cassette resulted in the reporter virus genome TB40-BAC4-IE-GLuc. Successfully recombined BAC genomes were checked for genome integrity by restriction fragment length analysis (RFLA) and sequencing of the region of interest. As both the fragment pattern in EcoRI/I-SceI double digests and sequence data from the inserted region showed the expected results, BAC-DNA was prepared and transfected into HFFs to reconstitute the virus. Within one week after transfection, foci of infected cells became visible and the virus grew to 100% cytopathic effect within two weeks. Supernatants from transfected cultures were analyzed with the Gaussia luciferase substrate coelenterazine to see whether functional luciferase was released by infected cells, which was the case with all tested samples.

Next, we checked the growth properties of the reconstituted virus TB40-BAC4-IE-GLuc, i.e. titers and cell tropism of progeny virus. As expected from our replacement strategy, TB40-BAC4-IE-GLuc grew to similar titers as the parental virus TB40-BAC4 (Fig. 1B) and maintained the broad cell tropism with high infection efficiency on fibroblasts, endothelial cells, and epithelial cells (Fig. 1C).

3.2. Detection of luciferase activity as a reporter for HCMV infection

While secretion of the GLuc into culture supernatants is desired for facilitated measurement of infection rates and viral growth, it also entails a problem: virus stocks are always “contaminated” with luciferase secreted into infectious supernatants during virus production. Particularly when cultures are exposed to high infection multiplicities, abundant background levels of “input” luciferase would mask de novo luciferase expression. Therefore, reduction of the “contaminating” luciferase in virus stocks is necessary to improve the signal-to-noise ratio. Attempts to remove input luciferase by repeated washing steps after the infection failed, because at least six washes were necessary until background signals were reduced below the detection limit, and this was not well tolerated by the cell cultures. Alternatively, infectious virus particles were pelleted by ultracentrifugation, supernatant containing the luciferase activity was discarded, and the pellet was resuspended in fresh medium. Two rounds of ultracentrifugation were sufficient to reduce contaminating luciferase by almost three log steps (Fig. 2A) without significant loss of infectivity (data not shown). One additional wash of cultures after incubation with the virus further reduced input luciferase levels about 50-fold resulting in a total reduction by more than four log steps (Fig. 2A; Supplementary protocol 1).

Using luciferase-depleted virus stocks, we then determined at which time point de novo luciferase activity could first be discriminated depending on the virus dose used for infection. Fibroblast cultures were infected with a 2-fold dilution series of virus stocks corresponding to infection multiplicities of 55–0.0008. At 2, 8, 16 and 24 h.p.i. 20 μl out of 150 μl supernatant was collected from each well and replaced by equal volumes of medium. Supernatants were stored at 4 °C and measured after the last collection time point (Fig. 2B). MOIs > 3.5 yielded discriminable luciferase signals already.
at 2 h.p.i. MOIs > 0.1 were detectable earliest at 8 h.p.i. Even MOIs down to 0.0008, corresponding to only 5 infected cells in one well of a 96-well plate, yielded luciferase responses clearly above background levels. This indicates that the reporter virus can be applied as a powerful tool to a variety of research questions.

Since TB40-BAC4-IE-GLuc was generated as a reporter virus to detect the initiation of viral gene expression, we next addressed the question of how luciferase signals correlate with the rate of infected cells as determined by indirect immunofluorescence detection of IE antigen-positive cells. HFFs were seeded into 96-well plates and infected with TB40-BAC4-IE-GLuc in 2-fold serial dilutions at MOIs ranging from 0.1 to 100. After 24 h, supernatants were collected and luciferase activity determined. The same cell cultures were then fixed and viral IE antigen was visualized by indirect immunofluorescence staining. Both assays correlated remarkably well until MOI = 6, showing the typical sigmoidal shape of dose-response curves (Fig. 2C). At higher virus doses, when rates of IE antigen-positive cells had already reached their maximum of 100%, there was still some further increase of GLuc intensities, indicating that the GLuc reporter can resolve differences at high infection multiplicities that would remain undetected by the immunofluorescence readout.

In order to achieve valid results, the concentration of the substrate coelenterazine is another important factor. At concentrations usually recommended by providers (10 μg/ml) luminescence could not be evaluated in most of the cases, because the upper detection limit was exceeded, whereas at coelenterazine concentrations of 0.2 μg/ml, almost all tested samples yielded evaluable signals. To determine the linear range of these signals, we analyzed a 2-fold dilution series of cell culture supernatant harvested at 24 h p.i., ranging from an undiluted supernatant to a 2048-fold dilution (Fig. 2D). Luminescence signals were linear over the complete range.

In conclusion, luminescence signals reflected luciferase activities in a linear manner over more than 3 log steps, and the luciferase readout truly reported the number of infected cells as validated by immunofluorescence detection of viral IE antigen. At high infection multiplicities the luciferase readout was even superior as compared to the immunofluorescence readout.

3.3. Application of the GLuc reporter virus for identification of hybridomas producing endothelial cell-specific neutralizing antibodies

TB40-BAC4-IE-GLuc was applied to screen a set of hybridoma cells for antibodies that would preferentially neutralize infection of endothelial cells rather than fibroblasts.

First, the serum from a mouse immunized with virions of a TB40-BAC-4 derived virus was analyzed for its 50% neutralization capacity in endothelial cells and fibroblasts (NC50HFF; NC50HEC; Supplementary protocol 2). NC50HFF values of 10−1.4 and NC50HEC values of 10−2.8 (corresponding to neutralization titers of 2500 and 630, respectively) were determined in three repeated tests, indicating a good reproducibility of the TB40-BAC4-IE-GLuc-based neutralization assay (Fig. 3A).

In the next step, supernatants from hybridoma cell cultures generated from B cells of this mouse were screened for their relative capacities to neutralize infection of endothelial cells versus fibroblasts (Supplementary protocol 3, Fig. 3B). From a total of 1880 hybridoma cultures, three cultures were selected because

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**Fig. 2.** Suitability of the GLuc expressing virus as a reporter of HCMV infection. (A) Reduction of unwanted luciferase background in fibroblast cultures at 24 h after infection with different conditions. Application of crude virus preparations resulted in high levels of input luciferase activity. Treatment of virus preparations by two rounds of ultracentrifugation (UC) and an additional medium exchange after infection (wash) reduced contaminating luciferase activity by more than four log steps. (B) Luciferase activity increased with higher multiplicity of infection (MOI) at each time point post infection (h.p.i.). Human foreskin fibroblasts were infected with serially diluted reporter virus TB40-BAC4-IE-GLuc. After 1 h, infectious supernatant was replaced by medium, reflecting the starting point of the experiment. At 2, 8, 16 and 24 h.p.i. 10% of supernatant was collected and replaced by an equal volume of medium. (C) Correlation of GLuc activity with the multiplicity of infection. HFF were infected with reporter virus TB40-BAC4-IE-GLuc in a 2-fold serial dilution at MOIs ranging from 0.1 to 100. After 24 h, supernatant of cell culture was collected and the luciferase activity was measured. (D) Linear dilution of luciferase supernatant leads to a linear decrease of luciferase signal. Two samples of cell culture supernatant, infected with TB40-BAC4-IE-GLuc, were serially diluted and luciferase activity was measured.
they had the highest ratio of neutralization in HECs versus HFFs. From all three cultures, individual clones were generated by single cell cloning via limiting dilutions. Two clones actually showed an endothelial cell-specific neutralization when their supernatants were analyzed in HECs versus HFFs using the GLuc-based neutralization assay (Supplementary protocol 2, Fig. 3C).

Taken together, the TB40-BAC4-IE-GLuc reporter virus is suitable for fast and easy screening of anti-HCMV hybridomas regarding their neutralization capacities in various cell types.

3.4. Application of the GLuc reporter virus for phenotypic characterization of BAC-cloned mutants during reconstitution

The Gaussia luciferase is special as its activity can be determined in the supernatant of living cultures and hence allows to quantify the expression of this reporter without the need to fix or lyse cells. The HCMV IE-promoter is known to induce high-level constitutive expression. Both combined could facilitate monitoring of viral growth at very low infection rates, e.g. during reconstitution of HCMV after transfection of BAC-cloned viral genomes. The idea is that the dynamics of viral growth is reflected by the increase in GLuc activity in the transfected cultures over time. If this applies, phenotypic differences between two viruses can be detected before the cytopathic effect within the culture is fully developed, thus speeding up and facilitating phenotypic characterization of targeted mutations introduced into BAC-cloned HCMV strains.

To test this concept, we compared the TB40-BAC4-IE-GLuc and a UL74stop mutant thereof in three independent transfections regarding the luciferase activity secreted into culture supernatants post transfection (p.t.). UL74 is not completely essential for viral replication but its disruption results in a small plaque phenotype. A UL74stop mutant was hence suitable to test whether monitoring of luciferase activity during the process of reconstitution can detect a partial growth deficit. In each experiment, 2 μg of BAC-DNA were transfected into HFF cultures using a commercially available calcium chloride transfection kit. In order to compensate varying transfection efficiencies, each transfection was performed in duplicates, which were pooled at 2 d.p.t. Starting from day 3 p.t., 150 μl aliquots of culture supernatants were drawn and stored for later measurement of luciferase activity (Supplementary protocol 4). In wild type transfected cultures, large foci of infected cells were formed within the first week after transfection and the cytopathic effect was complete mostly within the first two weeks after transfection. In contrast, in cultures transfected with TB40-BAC4-IE-GLuc-UL74stop only small foci were detectable throughout the experiments and it took about four weeks until the majority of the culture displayed a cytopathic effect.

This visual impression was reflected by clear differences in the kinetics of luciferase activity over time (Fig. 4A). In comparison, the mutant showed (i) an initial decrease in luciferase signals that was not apparent in wild type transfected cultures, (ii) a later onset of the increase in luciferase signals, (iii) and a smaller slope of the increase. The data were remarkably reproducible as indicated by the small standard errors of the mean, and the increase was linear when a logarithmic scale was applied to luciferase signals, thus allowing quantification of the slopes by linear progression.

To check whether the kinetics of the luciferase actually reflects growth of the virus, we additionally determined the infection rates by immunofluorescence staining of viral IE antigens. For this,
4. Discussion

Luciferase expressing reporter viruses have been used widely in the life science, including the study of cytomegalovirus replication and pathogenesis (Drouot et al., 2013; Dalal et al., 2009; Potzsch et al., 2011; Scrivano et al., 2011; Wiegens et al., 2015). However, an endotheliotropic cytomegalovirus expressing the secreted Gaussia luciferase (GLuc) was yet not available. Here, we provide a GLuc-expressing derivative of the endotheliotropic HCMV strain TB40-BAC4 and report how this virus can be utilized for screening approaches, e.g. (i) identification of hybridoma cells that produce neutralizing antibodies and (ii) quantitative analysis of BAC-cloned HCMV mutants regarding their growth properties directly during the reconstitution process.

Importantly, introduction of the GLuc expression cassette in place of the gpt gene within the BAC-vector sequence did not alter the phenotype of the recipient virus TB40-BAC4 regarding viral growth and cell tropism. The reporter virus TB40-BAC4-IE-GLuc grew to similar titers with similar kinetics as wild type virus, and it also has retained the broad cell tropism as indicated by efficient infection of endothelial cells. As intended by our cloning strategy, expression of GLuc under control of the major IE enhancer/promoter (MIEP) resulted in detection of secreted luciferase in the supernatant of infected cultures as early as 2 h after infection at infection multiplicities >10, which makes the virus suitable for assays regarding virus entry. The luciferase activity in the supernatant then increases until 24 h, and even at low infection multiplicities <0.01 luminescence can readily be detected at this time point, which is superior in our hands over other high-throughput readout systems like detection of IE antigen by ELISA (detection limit MOI = 0.1, data not shown). Due to expression under the control of the MIEP, it is not surprising that the luciferase readout reflects the rate of IE antigen-positive cells, which is a standard readout for infection efficiency. At high infection multiplicities >8, GLuc even appears superior, since a further increase of luminescence is still detectable when the rate of infected cells is already at the maximum and a quantitative increase of the amount of IE antigen/cell is hard to evaluate in immunofluorescence. Furthermore, when tested in a dilution series of a highly positive sample, the signal was linear over a range of at least 3 log steps, thus allowing for direct quantitative comparison of different mutants or conditions tested against each other in the same experiment.

One particular advantage of GLuc expressing reporter virus is that the luciferase-containing supernatant can be directly harvested from the living culture (Michelini et al., 2009), which enables kinetic analyses of an infected culture over extended time periods. In contrast, viruses expressing firefly or renilla luciferase need lysis of infected cells for harvest of the luciferase-containing material. As an example for possible applications we have shown here the direct monitoring of viral growth during the reconstitution of BAC-cloned virus after transfection of the respective DNA into HFFs. The slope of the growth curve indicates the replicative fitness of the respective mutant (Podlech et al., 2015), which allows fast and easy comparison of a set of mutants within few rounds of replication. This may be particularly relevant, when the development of compensating mutations during the reconstitution process might mask the phenotype of a mutation.

A reporter HCMV expressing the secreted Gaussia luciferase also provides certain advantages in the context of high throughput screening approaches. The fact that the signal is directly measured in the supernatant without any further manipulations like cell lysis, cell fixation or antibody incubations (i) reduces unwanted variation due to such procedures, (ii) avoids background problems due to nonspecific binding of antibodies and (iii) reduces the hands-on time as compared to an ELISA-based measurement by factor >4.5. As expression from the HCMV MIEP yielded a very strong signal,
concentrations of the substrate coelenterazine could be reduced to 0.2 μg/mL, thus rendering the costs for the substrate negligible, which is an important factor for high throughput screening approaches. As an example, we applied the reporter virus to the screening of hybridoma cells for antibodies that neutralize HCMV infection of endothelial cells better than fibroblasts. Other possible applications could be the screening of large drug libraries for novel HCMV-inhibiting agents.

In conclusion, an endothelialotropic HCMV reporter virus secreting Gaussia luciferase into the supernatant of infected cultures is now available for various applications that can facilitate both basic and translational research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2016.06.008.

References


2. A Two-step Screening Approach for the Identification of Blood Donors with Highly and Broadly Neutralizing Capacities against Human Cytomegalovirus

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Jessica Falk coordinated all experiments, neutralization assays were performed together with Martina Winkelmann and ELISA-based neutralization assays were done by Dagmar Stöhr. All further experiments were performed by Jessica Falk. Analysis, evaluation, visualization of data and conceptualization of the first manuscript draft were done by Jessica Falk. Reviewing and editing of the manuscript was done by Christian Sinzger and Ramin Lotfi.
A two-step screening approach for the identification of blood donors with highly and broadly neutralizing capacities against human cytomegalovirus

Jessica J. Falk, Martina Winkelmann, Hubert Schrezenmeier, Dagmar Stöhr, Christian Sinzger, and Ramin Lotfi

BACKGROUND: Hyperimmunoglobulins are frequently applied for prophylaxis and treatment of human cytomegalovirus (HCMV) infections but were only marginally effective in meta-analyses of clinical studies. This might be partially due to selection of donors rather than neutralizing capacities. To improve efficacy against HCMV infection, we aimed at developing a high-throughput screening method for identification of blood donors with highly and broadly neutralizing capacities.

STUDY DESIGN AND METHODS: Using a Gaussia luciferase-expressing reporter virus, 1000 HCMV immunoglobulin (Ig)G-positive plasma samples with known anti-HCMV IgG titers were analyzed regarding their neutralization titers against fibroblast and endothelial cell infection. Based on these results, a high-throughput screening was designed. Highly neutralizing plasma samples were further tested 1) by an enzyme-linked immunosorbent assay-based neutralization assay regarding efficiency against different HCMV strains and 2) for their efficiency compared to commercially available hyperimmunoglobulins.

RESULTS: Total anti-HCMV IgG titers did not correlate with neutralization. Mean neutralization capacities were 15-fold higher in endothelial cells compared to fibroblasts. All plasma samples neutralizing fibroblast infection were at least equally effective against infection of endothelial cells, providing the possibility to simplify our screening method by testing only fibroblasts as target cells with a plasma dilution of 1 in 400. Of the nine tested top HCMV neutralizers, four were broadly effective against different HCMV strains. All nine were significantly superior to hyperimmunoglobulins.

CONCLUSION: Donors with highly and broadly neutralizing capacities can be identified by a two-step high-throughput screening approach. This may provide a basis for improved antibody-based treatment or prophylaxis of HCMV infections.

H uman cytomegalovirus (HCMV) is a ubiquitously prevalent pathogen causing acute and latent infections in its host. In immunocompetent individuals infections with HCMV are generally mild to nonsymptomatic. However, an HCMV infection may be life-threatening in immunocompromised patients or lead to innate disabilities upon intrauterine infection. Depending on comorbidities or concomitant conditions (e.g., pregnancy) some patients may not be eligible for effective treatment with available virostatic drugs due to associated side effects. Passive immunization with human-derived hyperimmunoglobulins is considered as an alternative even though immunoglobulins have been only marginally effective in clinical trials. Given the observations that immunocompetent individuals are able to keep the infection and reactivation of virus in check and that virus dissemination can be restricted by anti-HCMV treatment with hyperimmunoglobulins is

ABBREVIATIONS: BAC = bacterial artificial chromosome; HCMV = human cytomegalovirus; HFF(s) = human foreskin fibroblast(s); IE = immediate early.

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promising in principle, but seems to need further refinement. Considering that some CMV-positive pregnant women with high titers of antibodies against HCMV cannot prevent transplacental viral transmission to the fetus, it seems that there are qualitative differences between anti-HCMV. The serologic immune response against HCMV can show differences in its neutralizing capacity depending on the virus strain being on hand and the cell type that is infected. This is on the one hand due to the genotypical diversity of the viral glycoproteins that can lead to different recognition sites for the immune system and on the other hand caused by the two entry mechanisms for different cell types. HCMV can infect nearly all cell types in the body. In case of virus transmission through oral mucosa or placenta, it is possible that the epithelial cells play an important role, while white blood cells and endothelial cells could be the primary targets after transplantation of hematopoietic stem cells or blood transfusion. The infection of fibroblasts might enable viral spread to the body tissues. HCMV uses two different gH-glL-containing glycoprotein complexes to enter its target cells. The trimeric gH-glL-glO complex is necessary for efficient entry in all cell types tested so far. In addition, certain cell types including endothelial cells and epithelial cells need the pentameric gH-glL-pUL128-pUL130-pUL131A complex. Other cell types allow infection independent of the pentamer, and fibroblasts are the prototypical cell type for this entry pathway. With regard to neutralization, anti-HCMV differ in terms of their ability to protect different target cells from infection, that is, neutralizing antibodies directed against the pentamer inhibit only infection of endothelial and epithelial cells but do not protect fibroblasts. To investigate neutralizing capacities of different antibodies against HCMV, an HCMV strain with a broad cell tropism is necessary. It should be able to infect endothelial cells and fibroblasts as they are the prototypical cell types for the two different entry routes. While clinical isolates immediately after cultivation in fibroblasts express both gH-glL complexes, they usually lose the pentamer within a few passages. Due to its selection on endothelial cells, HCMV strain TB40-BAC4 still expresses the pentamer, albeit at lower levels. This modification may reduce the selective pressure against the respective gene while still mediating the broad cell tropism. The genome of this strain is available as a bacterial artificial chromosome (BAC) with a genetically stable pentamer gene region. Recently, a Gaussia luciferase-expressing derivative of this strain has been generated (TB40-BAC4-IE-GLuc) that is particularly suitable as a reporter for HCMV infection in high-throughput analyses.

MATERIALS AND METHODS

Cells, viruses, and plasma

HEC-LTT is a conditionally immortalized cell line derived from primary human umbilical vein endothelial cells that were shown to be a useful in vitro model for endothelial cell infection by HCMV. HEC-LTTs were cultured in endothelial growth medium (EGM BulletKit, Lonza), supplemented with 2 μg/mL doxycycline. Human foreskin fibroblasts (HFFs) were grown in MEM5 (MEM with 5% fetal bovine serum, GlutaMAX [Life Technologies], and 100 μg/mL gentamicin) supplemented with 0.5 ng/mL basic fibroblast growth factor (Life Technologies). During experiments, both HFFs and HEC-LTTs were kept in MEM5. Endothelial cells were cultured in vessels and microlifts coated with 0.1% gelatin (Sigma-Aldrich). All virus stocks were used cell free. The supernatants of infected cell cultures were therefore centrifuged at 3220 × g for 10 minutes to remove cell debris. AD169 (available at ATCC; VR-538), Towne (available at ATCC; VR-977), Toledo (provided by S. Plotkin), and Merlin (available at ATCC; VR-1590) are widely used HCMV strains, but lack the pentameric complex and are therefore nonendotheliotropic. TB40/E, VR18144 (provided by G. Gerna) and VHL/E (provided by W. Waldman) express the pentameric complex and represent endotheliotropic HCMV strains. HCMV strain TB40/E is the parental virus from which the reporter virus TB40-BAC4-IE-GLuc was cloned. The TB40-BAC4-IE-GLuc expresses Gaussia luciferase immediately after infection of target cells, was used for the luciferase-based assays (TB40/E and its derivative are available on request to C. Sinzger). To reduce background luciferase activity, cell-free supernatants of the reporter virus were ultracentrifuged twice in a SW28 rotor at 23,000 U/min (100,000 × g) before being stored in aliquots at −80°C.

EDTA-treated plasma samples from anti-HCMV-positive blood donors were provided by the German Red Cross Blood Transfusion Service Baden-Württemberg-Hessen. The project has been approved by the Ethical Committee of the University of Ulm (Votum No. 53/14).

Quantitative analysis of anti-HCMV

Anti-HCMV titers of the first 1000 plasma samples were measured in the Institute for Transfusion Medicine using a CMV and IgG + IgM test (Enzygnost, Siemens Healthcare) in an endpoint dilution approach. These data were used to analyze the correlation of neutralizing capacities with anti-CMV levels.

To adjust selected plasma samples and commercially available hyperimmunoglobulins regarding their anti-HCMV for a subsequent comparison of their neutralizing capacities, a quantitative test (CMV-IgG-ELISA PKS, Medac) was applied that directly indicated anti-HCMV IgG concentrations as arbitrary units (AU) per mL.

Gaussia luciferase-based neutralization assay

To evaluate the HCMV-neutralizing capacities of plasma samples in a high-throughput fashion, the GLuc-
expressing reporter virus TB40-BAC4-IE-GLuc was employed, which allowed us to determine the extent of infection in a partially automated setting with good reproducibility. Only plasma but not serum was routinely available from our blood donors. As incubation of virus with native plasma regularly resulted in coagulation, which impeded exact pipetting, we had to remove clotting factors before neutralization tests. To achieve this, plasma samples were recalcified by mixing one part of the plasma sample with nine parts of MEM5 and incubating the mixture for at least 2 hours at 37°C, followed by storage at 4°C for up to 2 days.

For the neutralization assay, HFFs or HEC-LTTs were seeded the day before the experiment in 96-well plates at a density of 1.5 × 10^4 per well and allowed to attach overnight. The reporter virus TB40-BAC4-IE-GLuc (MOI = 1) was added to a serial dilution of each recalcified plasma sample. The virus-plasma mixture was incubated for 2 hours at 37°C. The medium of target cells was replaced with the virus-plasma mixture and incubated for an additional 2 hours at 37°C followed by further replacement with an appropriate volume of suitable medium for each cell type. After an additional 24 hours of incubation, 20 μL of cell culture supernatant was transferred to a 96-well plate (Greiner Bio-One). As a substrate, 50 μL of coelenterazine (PJK GmbH) at 0.2 μg/mL in phosphate-buffered saline supplemented with 5 mmol/L NaCl was added automatically to each well right before starting the measurement. Light signals emitted by the luciferase reaction were measured using a luminescence plate reader (Chameleon, Hidex) within 2 seconds and were given as relative light units (RLU).

The signal of the luciferase activity was normalized separately for each experiment in reference to the maximal infection: the luciferase activity that reflects the maximal infection was determined by calculation of the geometric mean of the luciferase signals for the highest dilution step (1/20,480) of all plasma samples in one experiment, as we had seen in previous experiments that neutralization never occurred at this dilution. To control for the performance of the Gaussia luciferase assay, positive and negative controls were included, that is, the reporter virus was mixed with an HCMV-seropositive neutralizing serum and an HCMV-seronegative nonneutralizing serum.

**ELISA-based neutralization assay**

To evaluate whether the plasma samples with the highest neutralizing potential against HCMV strain TB40-BAC4-IE-GLuc also had a broadly neutralizing activity against other HCMV strains, they were retested using a collection of endotheliotropic and nonendotheliotropic HCMV strains that represent almost all known genotypes of the various HCMV envelope glycoproteins (for details see Table S1, available as supporting information in the online version of this paper). Since these strains were not available as Gaussia luciferase reporter viruses, neutralization was tested using immunodetection of viral immediate early (IE) antigens. To facilitate the quantitative evaluation, IE antigens were detected with an ELISA readout essentially as previously reported.

Briefly, cell-free virus preparations were incubated with recalcified plasma samples for 2 hours as described in the previous section for the Gaussia luciferase-based assay. HFFs were then incubated with these virus-plasma mixtures in duplicates for 24 hours and fixed afterward with 80% acetone (Sigma Aldrich) for 5 minutes at room temperature. Fixed cells were then incubated for 90 minutes at 37°C with 1:1000 diluted anti-HCMV IE antigen antibody (E13, Argene). After several washing steps to remove unbound antibodies, a horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse IgG-HRP, Santa Cruz Biotechnology) was diluted 1:400 and added for 45 to 60 minutes at 37°C. Ortho-Phenylenediamine in peroxidase buffer (Thermo Fisher Scientific) served as HRP substrate. The reaction was stopped by adding 1 mol/L sulfide acid. Optical density (OD) was measured at 493 nm, and mean values of duplicates were used for further calculations. All mean values were corrected by subtracting the OD values obtained with mock infections (applying plasma without virus) and these background-corrected values were then used for further calculations (see section “Evaluation of neutralization capacity”). Incubation of cells with virus only was performed to determine the maximal infection signal. To control for the performance of the ELISA assay, positive and negative controls were included, that is, each virus strain was mixed with an HCMV-seropositive neutralizing serum and an HCMV-seronegative nonneutralizing serum.

**Evaluation of neutralizing capacity (NT50) from neutralization rates**

Irrespective of whether infection signals were obtained by Gaussia luciferase-based assays (RLU) or by ELISAs (OD), relative infection rates were determined by dividing the corrected signals obtained with virus-plasma mixtures by the maximal infection signal

\[
\left( \frac{\text{Signal(virus+plasma)}}{\text{Signal(max)}} \right).
\]

Neutralization rates were calculated by subtracting the relative infection rate from 1:

\[
\left( 1 - \frac{\text{Signal(virus+plasma)}}{\text{Signal(max)}} \right).
\]

Neutralization rates were plotted against plasma concentrations and the dose-response curves were analyzed by
nonlinear regression. The plasma concentration at which 50% of the target cells were protected from infection (neutralization rate, 0.5) is referred to as the 50% neutralization concentration (NC50). The reciprocal of the NC50 is the neutralization titer (NT50). The NT50 describes the plasma dilution factor that yields half-maximal neutralization. The neutralizing capacity of a plasma is represented by its NT50.

**Gaussia luciferase-based screening for high neutralizers of fibroblast infection**

For the high-throughput screening approach, HFFs were seeded on a 96-well plate at a concentration of $1.5 \times 10^4$ cells/well. To remove clotting factors within the plasma, one part of each sample was incubated with 99 parts of MEM5 for at least 2 hours at $37^\circ C$, followed by storage at $4^\circ C$ for up to 2 days. On each 96-well plate, one aliquot of the reference plasma was included. The reference plasma was defined by a NT50HFF of 2400 as determined in the Gaussia luciferase-based neutralization assay. All samples were then further diluted with preparations of the reporter virus TB40-BAC4-IE-GLuc to obtain a final plasma dilution of 1:400 and an MOI of 1. The mixture was incubated for 2 hours at $37^\circ C$ to allow for neutralization and then added to the cell cultures for an additional 2 hours at $37^\circ C$ (one sample per plasma, no replicates). The mixture was removed and replaced with MEM5 for an overnight incubation. The next day, luciferase activity was measured.

**Comparison of commercially available hyperimmunoglobulin and top neutralizing plasma samples regarding their neutralizing capacities**

The anti-HCMV IgG concentrations of a commercially available hyperimmunoglobulin product (Cytotect, Biotest) and nine different highly neutralizing plasma samples were determined using a quantitative ELISA (CMV-IgG-ELISA PKS, Medac). The IgG concentrations were given in AU/mL, facilitating adjustment of IgG concentrations for comparison. All plasma samples were diluted 100 times in MEM5. The anti-HCMV IgG concentration of the hyperimmunoglobulin preparation was individually adjusted to the anti-HCMV IgG concentration of each 100-fold diluted plasma. The adjusted antibody preparations were mixed with reporter virus TB40-BAC4-IE-GLuc at an estimated MOI of 2.5. After incubating the mixture for 2 h at $37^\circ C$, it was added to HFFs for a further 2 h at $37^\circ C$. The antibody-virus mixture was then removed and replaced by MEM5 for an overnight incubation. Finally, supernatant was collected from each well and the luciferase activity was measured.

The luciferase activity of a CMV-seronegative plasma was defined as the signal of maximal infection. The data were normalized to the maximal infection and subtracted from 1 to obtain the neutralization. Neutralization was given as the percentage of the maximal infection.

**RESULTS**

The neutralizing capacity is not reflected by the concentration of anti-HCMV in plasma samples from HCMV-seropositive donors

The currently available hyperimmunoglobulin preparations are selected according to their antibody titer under the assumption that this could serve as criterion for virus neutralization. To evaluate this assumption, we analyzed plasma samples from HCMV-seropositive blood donors for the correlation between anti-HCMV titers and anti-HCMV-neutralizing capacities. Total anti-HCMV titers, including IgG and IgM, were determined by endpoint dilutions using a commercially available ELISA test (CMV/IgG + IgM, Enzygnost, Siemens Healthcare). Neutralizing capacities of plasma samples were tested by a neutralization assay based on a luciferase reporter virus. The neutralizing capacity against HCMV infection is given by the half-maximal neutralization titer (NT50), which indicates the dilution factor of plasma that is necessary to protect 50% of target cells from HCMV infection. Testing 1000 plasma samples and plotting their anti-HCMV immunoglobulin titers against their NT50s, we observed a significant ($p < 0.0001$) but very weak correlation for both tested target cell lines. The coefficient of determination $r^2$ was 0.04 for fibroblasts and 0.10 for endothelial cells, which means that only 4% of the variation in the neutralization titers in HFFs and 10% of the variation in HECs are explained by the difference in the overall anti-HCMV immunoglobulin titers. Thus, we could demonstrate that for both target cells, the anti-HCMV titer does not reflect the capacity of the plasma to protect from HCMV infection (Fig. 1). This discrepancy was most obvious for plasma samples with low anti-HCMV titers. For example, plasma samples with an HCMV titer of 16 showed NT50 values ranging from 10 to 6800 for fibroblast infection (Fig. 1A). For the anti-HCMV titer of 32, the endothelial cell specific neutralization titers ranged from 330 to 48,000 reflecting a more than 120-fold difference (Fig. 1B). A broad variance of NT50s was also seen at all further HCMV titer levels and with both cell types. Even if there was a weak correlation between HCMV titers and neutralization titers of plasma samples, this fact is extenuated by the strong NT50 variations within the distinct HCMV titer levels.

**High fibroblast-specific neutralization titers can serve as an indicator for overall high neutralization capacities**

Based on our observations that the anti-HCMV titer did not reflect the antiviral activity of the plasma samples, we
focused on developing a feasible screening tool for assessing the neutralizing capacities of the respective plasma samples. An important aspect was the comparison between endothelial cells and fibroblasts as target cells for infection. Therefore, we compared the NT50s of 1000 plasma samples in the two different cell types, with endothelial cells serving as a model for gH-gL-pUL128-pUL130-pUL131A pentamer-dependent entry and fibroblasts representing pentamer-independent entry.

Plotting the data in a frequency distribution shows that the vast majority of plasma samples had NT50s between 100 and 1000 when using fibroblasts as target cells, whereas endothelial cell infection was neutralized more efficiently, with NT50s between 2000 and 10,000. The peak of the distribution is at 6000 for endothelial cells and at 400 for fibroblasts, and there is relatively little overlap between the two histograms (Fig. 2). This means that 15-fold more plasma was necessary to achieve equal neutralizing efficiencies in fibroblasts compared to endothelial cells.

Analyzing the data in a dot plot allows for comparing the neutralization capacities for individual plasma samples as each dot represents one plasma sample (Fig. 3). It became obvious that NT50s for endothelial cells and fibroblasts are not randomly distributed but show a distinct pattern. The majority of dots form a cloud, indicating that most HCMV-seropositive samples can neutralize both infection of endothelial cells and fibroblasts. The axis of the cloud reaches from the lower left to the top right. Its direction reflects the fact that plasma samples with higher titers against fibroblast infection also have higher titers against endothelial cell infection, with a correlation coefficient of 0.37. Importantly, all plasma samples with high neutralizing capacities against fibroblasts (NT50 > 2200) also neutralized endothelial cell infection equally well or even better (Fig. 3). Since our aim was to find plasma samples with high neutralizing capacities against both cell types, this finding allowed us to focus on the identification of plasma samples with exceptionally high neutralizing capacities for fibroblast infection. The top 2.5% neutralizers are defined as those showing NT50s higher than...
mean plus two times standard deviation of all NT50s. These top neutralizing plasma samples can be diluted more than 2000-fold and still achieve 50% reduction of infection in fibroblasts. Without exception, these top neutralizers of fibroblast infection were also highly effective against endothelial cell infection. Based on this analysis, we selected a reference plasma with an NT50 of 2400, representing the “cutoff” for identification of plasma samples with exceptional neutralization capacities against both cell types. Using this reference plasma, a simplified screening procedure was established using just one dilution step and only fibroblasts as target cells (see below).

High-throughput screening with an HCMV reporter virus identifies exceptionally high neutralizing plasma samples from anti-HCMV-seropositive individuals

Based on our results, we performed a simplified screening method for identifying plasma samples with exceptionally high neutralizing capacity against HCMV. For this purpose, we used only one target cell line (fibroblasts) and just one plasma dilution according to our cutoff. A plasma dilution of 1 in 400 was chosen, which enabled us to clearly discriminate between average plasma samples and the exceptional ones. The assessed neutralizing activity of each investigated plasma sample was further normalized to one particular reference plasma (NT50HFF = 2400), resulting in a distinct neutralizing performance value. A new set of 1000 plasma samples was investigated according to this procedure and the distribution of their neutralizing performances was plotted (Fig. 4A). Since the reference plasma represents the cutoff, all plasma samples with equal or higher neutralizing performance compared to the reference plasma (performance of ≥1) were considered top neutralizers. Within this data set of 1000 plasma samples, 36 samples showed top neutralizing performances. To prove the assumption that all top neutralizers identified in this screening on fibroblasts also have high neutralizing capacities against endothelial cell infection, the NT50 values of nine plasma samples were exemplarily tested for infection of both cell types. As expected, all of them showed equal or higher NT50s for endothelial cells than for fibroblast infection (Fig. 4B).

The highly neutralizing plasma samples can be further characterized according to their capability to neutralize different HCMV strains

The surface glycoproteins of different HCMV strains differ in their amino acid sequence, which may result in a variable epitope pattern of the viral glycoproteins. Since the glycoproteins mediate the entry of the virus into the target cells, the neutralizing efficacy of plasma may be strain specific.4,10,41 Thus, depending on the recognition site, even an anti-HCMV belonging to the highly neutralizing ones may have a rather “narrow” neutralizing capacity recognizing just one or few viral strains. For this reason, the identified top neutralizing plasma samples that had performed well against the TB40/E-derived reporter virus were further tested for their broadness of neutralization against seven different HCMV strains. As these strains did not express the luciferase reporter, we applied a neutralization assay that relies on detection of viral IE antigens by an ELISA readout, thus allowing for the analysis of various HCMV strains without the need for a reporter gene. Fibroblasts were infected with the individual HCMV strains in the presence of the plasma samples and the strain specific half-maximal neutralization titers (NT50s) were assessed (Fig. 5). Plasma samples that achieved NT50s higher than 100 against all viral strains were regarded “broadly neutralizing.”

Of nine tested top neutralizing plasma samples, four were found to be broadly neutralizing. These broadly neutralizing plasma samples could be diluted 100-fold and
still reduced infection of seven viral strains to the half. As the parental TB40/E strain was also included in the set of HCMV strains, all plasma samples identified as highly neutralizing against TB40-BAC4-IE-GLuc in the luciferase-based screen were retested by an independent method. All nine top neutralizers were confirmed to be highly effective against strain TB40/E. In conclusion, by using our simplified screening procedure we were able to identify top neutralizing plasma samples in a high-throughput fashion and, additionally, approximately half of them also showed broad effectiveness against fibroblast infection by different virus strains.
Highly neutralizing plasma samples are superior to conventional hyperimmunoglobulin preparations in vitro

To compare the human plasma samples with commercially available hyperimmunoglobulin preparations, HCMV-specific antibody (anti-HCMV IgG) concentrations were determined and adjusted. In view of the future possibility of applying the human plasma directly for treatment, the plasma samples were used at 100-fold dilution as this was the expected dilution in vivo. Hence the anti-HCMV IgG concentration of the hyperimmunoglobulin preparation was adjusted to 1% of the anti-HCMV IgG concentration of each single plasma sample for a pairwise comparison. Consequently, the applied anti-HCMV IgG concentration of each individual plasma sample was equal to the corresponding hyperimmunoglobulin preparation. By this approach, nine of the top neutralizing plasma samples were directly compared to the hyperimmunoglobulin. Their neutralization efficacy was investigated with the luciferase reporter virus assay and is represented by the percentage of neutralization. This was determined by comparing the luciferase activity of each preparation to the maximal infection level, reflected by a seronegative plasma (Fig. 6). Adjusted to the anti-HCMV IgG concentration, all nine selected plasma samples showed higher neutralization than their corresponding hyperimmunoglobulin preparations. When all tested plasma samples and the corresponding adjusted hyperimmunoglobulin preparations were analyzed by a Wilcoxon rank-sum test for matched pairs, the set of plasma samples achieved a mean neutralization of 89%, whereas the mean neutralization with the hyperimmunoglobulin was 52%. The identified top neutralizers were significantly (p < 0.001) more effective against infection of fibroblasts than conventional hyperimmunoglobulin preparations.

DISCUSSION

The established screening method offers a straightforward process to identify plasma samples that are superior to the currently available hyperimmunoglobulins, regarding their neutralization of HCMV. Aiming to identify plasma antibodies with the highest protective effect against HCMV infection, we focused on the neutralizing capacity of plasma rather than on the mere anti-HCMV titer that—as we could show—did not serve as a reliable surrogate marker for HCMV neutralization. Consistent with published data, we observed that a much higher concentration of antibody is required to neutralize HCMV infection of fibroblasts compared with infection of endothelial cells. Thus, screening for top neutralizers could be simplified by restricting the assay to fibroblasts. The cell type-dependent differences in neutralizing capacities can be attributed to the fact that HCMV entry into those cell types is mediated by two distinct glycoprotein complexes. While for fibroblast infection the trimeric complex (gH/gL/gO) is sufficient, entry into endothelial cells requires the pentameric complex (gH/gL/UL128-131) in addition. Since there is in general a huge portion of neutralizing antibodies against the pentamer and no known monoclonal against the trimer, it seems that the trimeric complex may not be as accessible for an effective immune response and for antibody binding as the pentamer. Based on this notion, current neutralization assays for diagnostic purposes focus on NC50 against endothelial/epithelial cell infection given its higher sensitivity. However, the sensitivity of the test is of minor importance when aiming to identify potent anti-HCMV.

We also found that in about half of the cases the protective capacity of highly neutralizing antibodies did not meet our strict criteria of a broad response, that is, neutralization of all tested virus strains at NT50s of more than 100. The finding of strain-specific neutralization by sera from HCMV-seropositive individuals has been reported...
repeatedly.\textsuperscript{9,40,41} Hence, it is not surprising that there is variation among our donors regarding the broadness of their response. Our stringent selection criteria may explain why the frequency of “broadly neutralizing” donors appears relatively low. Consequently, the efficacy of an antibody preparation against several different HCMV strains should be considered to a much greater extent when selecting plasma donors for the production of anti-HCMV immunoglobulins. The term “broad efficacy” might implicate two distinct and nonoverlapping characteristics of an antibody, 1) its neutralizing capacity against a broad range of virus strains but also 2) its protective capacity against infection of different types of target cells, endothelial/epithelial cells, and fibroblasts. It is noteworthy that all top neutralizers identified in the fibroblast screen were also highly effective on endothelial cells whereas the reverse does not apply. Moreover, these criteria should go hand in hand with a low amount of antibody needed to achieve maximum efficacy. Based on our results we provide a high-throughput method to identify antibodies with broad efficacy against HCMV infection, according to the above-mentioned definition. Our two-step approach is a simple reporter virus-based screening for the identification of highly neutralizing plasma samples followed by an ELISA to test for the broadness of the response against several different HCMV strains (Fig. 7). It could be shown that the highly neutralizing plasma samples selected by the screening were superior to the commercially available hyperimmunoglobulin preparations in neutralizing fibroblast infection. This indicates an enhanced efficacy of our qualitatively selected plasma compared to previously used strategies.

Beyond the aspect of neutralization addressed in this study, it might also be desirable to identify donors that can restrict cell-associated growth, which by definition is resistant to neutralizing anti-HCMV. A more comprehensive screening approach might be able to identify exceptional donors that would inhibit such cell-associated growth. This is, however, beyond the scope of this study that is focused on improving the selection of donors with neutralizing activity against cell-free virus, and it will require a different novel assay system enabling high-throughput testing of plasma samples regarding inhibition of cell-associated spread.

In conclusion, our screening approach allows us to identify top neutralizing donors for a passive immunization against HCMV in situations where transmission or spread via cell-free virus occurs, that is, dissemination in immunocompromised patients and intrauterine infection. We plan to study plasma transfusions from such donors in a clinical trial.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1. Glycoprotein-genotypes of selected human cytomegalovirus strains.
3. Identification of Elite Neutralizers With Broad and Potent Neutralizing Activity Against Human Cytomegalovirus (HCMV) in a Population of HCMV-Seropositive Blood Donors

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Jessica Falk coordinated all experiments. Neutralization assays were performed together with Martina Winkelmann and ELISA-based neutralization assays were done by Dagmar Stöhr. All further experiments were performed by Jessica Falk. Analysis of data, evaluation and visualization by Jessica Falk. Conceptualization of the first manuscript draft was done by Jessica Falk. Reviewing and editing of manuscript was done by Christian Sinzger and Jessica Falk.
Identification of Elite Neutralizers With Broad and Potent Neutralizing Activity Against Human Cytomegalovirus (HCMV) in a Population of HCMV-Seropositive Blood Donors

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To improve the potency of anti-human cytomegalovirus (HCMV) immunoglobulin preparations, we intended to find elite neutralizers among 9000 HCMV-seropositive blood donors. We identified the top 2.6% neutralizers by use of high-throughput screening and further analyzed the 80 neutralizers with the most effective plasma for strain-independent activity. Of those, 58 had broad neutralizing activity against various HCMV strains and hence were regarded as elite neutralizers. All elite neutralizers were then analyzed to determine their effect on individual virus particles during entry. Most had plasma specimens that preferentially inhibited viral penetration, whereas 2 had exceptional plasma specimens that prevented adsorption of virus to cells. Furthermore, the neutralizing capacity of plasma samples from 3 randomly chosen elite neutralizers was up to 10-fold higher than that for commercial immunoglobulins. In a retrospective analysis of 6 selected donors, anti-HCMV neutralization titers in repeated donations were constantly high over 5 years. In conclusion, plasma samples from elite-neutralizing donators can be considered to improve antibody-based treatment of HCMV infections.

Keywords. Cytomegalovirus; hyperimmunoglobulins; neutralizing antibodies; elite neutralizers; blood donors.

Human cytomegalovirus (HCMV) is a ubiquitously distributed herpesvirus. After primary infection, it causes lifelong latency, and reactivation can result in episodes of viral replication. Primary infection of immunocompetent individuals can cause mild symptoms or mononucleosis-like disease. Reactivations and reinfections are usually asymptomatic. In contrast, immunocompromised individuals, such as patients with AIDS and transplant recipients, can experience severe complications due to primary or reactivated infection. The risk for HCMV infection after transplantation of solid organs or hematopoietic stem cells is about 50%, and adverse outcomes may include graft failure, graft-versus-host disease, and increased susceptibility to other infections [1–4]. Intrauterine HCMV infection occurs at a prevalence of 0.3%–1.5% and is a leading cause of congenital disabilities [5, 6], including hearing loss and other neurological impairments [7, 8]. It was generally assumed that primary infection during pregnancy is more hazardous than reactivation or reinfection, owing to protective effects of preexisting maternal antibodies, but this assumption has been questioned recently after synopsis of the available information [9].

It is obvious that high levels of HCMV-specific antibodies in diagnostic assays are not sufficient for protective immunity [9–11]. In line with this, reports on the impact of HCMV hyperimmunoglobulins in pregnant women with primary HCMV infection [12–14] and in recipients of transplants [1, 15–17] are controversial. In principle, anti-HCMV immunoglobulins were found to be beneficial in transplant recipients, but the effects on morbidity and mortality were only moderate. Direct-acting antivirals such as ganciclovir or foscarnet are effective by targeting the viral polymerase, but their use may be complicated by myelotoxic or nephrotoxic side effects. The recently approved terminase inhibitor letermovir is apparently less toxic but may also be limited by the development of resistance [18]. Therefore, a potent passive immunization strategy is still a desirable alternative. In a recent clinical trial, a combination of 2 neutralizing anti-HCMV antibodies reduced the frequency of HCMV viremia [19, 20]. Interestingly, these antibodies were shown to act mainly via neutralization but not via antibody-dependent cellular cytotoxicity or complement.
As an approach to improve the quality and efficacy of anti-HCMV immunoglobulin preparations, we screened a large population of blood donors for their neutralizing capacities [21]. On the basis of our findings, we identified elite neutralizers, defined as individuals whose plasma is more potently neutralizing than currently available HCMV hyperimmunoglobulins. The concept of elite neutralization was developed in the field of human immunodeficiency virus (HIV) research, where it describes a small subset of HIV-1–infected individuals who generate high titers of broadly neutralizing antibodies [22, 23]. Elite neutralizers are not identical with elite controllers, who limit viral replication, often in the absence of broadly neutralizing antibodies [24].

Broad and potent antibodies were cloned from elite neutralizers to improve passive immunization strategies against HIV [25], and the same concept was applied to other viruses [26]. Regarding HCMV, the breadth of neutralizing antibodies concerns not only the inhibition of various HCMV strains but also the efficacy against cell type–specific entry routes mediated by different viral glycoprotein complexes. While antibodies of seropositive individuals usually have a high neutralization capacity against infection of endothelial and epithelial cells via the pentamer gH/gL/pUL128/pUL130/pUL131A, infection of fibroblasts via the trimer gH/gL/gO is less efficiently neutralized [21, 27]. This may also be relevant in the context of intrauterine infection, as trophoblast progenitor cells were protected against HCMV infection by antibodies against gB but not by anti-pentamer antibodies [28].

We therefore investigated plasma specimens from blood donors to identify elite neutralizers with exceptional strain-independent neutralizing activity against infection of both cell types. This could provide the basis for an improved passive immunization strategy for patients endangered by HCMV, including both direct use of plasma preparations and the isolation of monoclonal antibodies from such donors.

**MATERIALS AND METHODS**

**Cells, Viruses, Plasma Samples, and Antibodies**

Human foreskin fibroblasts (HFFs) were grown in minimal essential medium with 5% fetal bovine serum (MEM5; GlutaMAX, Life Technologies) and 100 μg/mL gentamicin supplemented with 0.5 ng/mL basic fibroblast growth factor (Life Technologies). Conditionally immortalized human endothelial cells (HEC-LTTs) [29, 30] were cultured in vessels and microplates coated with 0.1% gelatin (Sigma-Aldrich) in endothelial growth medium (EGM BulletKit, Lonza), supplemented with 2 μg/mL doxycycline. During experiments, HFFs and HEC-LTTs were both kept in MEM5.

HCMV strains AD169 [31], Towne [32], Toledo [33], Merlin [34], VR1814 [35], VHL/E [36], and TB40/E [37] and derivatives thereof [38] were used in this study. This panel was chosen as it covers almost all of the established genotypes of HCMV envelope glycoproteins [21]. Viral stocks were generated by centrifuging supernatants of infected cell cultures at 3220 g for 10 minutes to remove cell debris.

Plasma samples from HCMV-seropositive blood donors were provided by the German Red Cross Blood-Transfusion Service, Baden-Württemberg and Hessen, with informed consent according to human experimentation guidelines (Ethical Board of Ulm University vote number 53/14). Remnant clotting factors were removed by recalcification prior to use in neutralization tests [21].

The immunoglobulin G (IgG) concentration in plasma, in grams/liter, was determined by a turbidimetric assay (Cobas 8000 c502; Roche). Anti-HCMV IgG concentrations were determined as arbitrary units (AU) per milliliter (CMV IgG enzymelinked immunosorbent assay [ELISA] with a pipetting-control system; Medac, Hamburg).

Antibodies (IgGs) were purified from plasma samples by using protein A chromatography and were dialyzed against phosphate-buffered saline (PBS) as previously described [39].

**Gaussia Luciferase-Based Screening for High Neutralizers of Fibroblast Infection**

HFFs were seeded on 96-well plates at $1.5 \times 10^4$ cells/well. Plasma samples were mixed with the Gaussia luciferase–expressing reporter virus TB40-BAC4-IE-GLuc to obtain a final plasma dilution of 1:400 and a multiplicity of infection (MOI) of 1. The mixture was preincubated for 2 hours at 37°C and added to cells to initiate infection. After cells were incubated for 2 hours at 37°C, the mixture was replaced by MEM5. Cell cultures were then incubated overnight, 20 μL of supernatant of each infected cell culture was mixed with coelenterazine (0.2 μg/mL in PBS supplemented with 5 mmol/L NaCl; PJK), and luminescence signals (measured as relative light units [RLU]) were measured with a microplate reader (Chameleon; Hidex). The neutralizing performance was determined as the ratio of the RLU for reference plasma to the RLU for test plasma.

**Determination of Half-Maximal Neutralization Titer (NT_{50}) by an ELISA-Based Neutralization Assay**

HFFs or HEC-LTTs were seeded in 96-well plates at $1.5 \times 10^4$ cells/well. Plasma samples were serially diluted in duplicates, mixed with TB40/E (MOI = 1), and preincubated for 2 hours at 37°C. The virus-plasma mixtures were incubated with cells overnight at 37°C. The cells were then fixed with 80% acetone (Sigma Aldrich) and stained with a monoclonal antibody against HCMV immediate-early antigen (E13; Argene Biosoft) [40] and a secondary antibody (goat anti-mouse IgG) conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Substrate (ortho-phenylenediamine; Thermo Fisher Scientific) was added for 30 minutes, the reaction was stopped with 1 M sulfuric acid, and the OD₄₉₀ was measured. Mock values (ie,
signals for plasma without virus) were subtracted from mean values of duplicates, and the background-corrected values (ie, the signal for virus plus plasma) were divided by the maximal signal (ie, signal for virus only) to determine the relative infection rates. Neutralization rates were calculated as $1 - ([\text{signal for virus plus plasma}] / [\text{signal for virus only}])$, and the corresponding dose-response curves were analyzed by nonlinear regression. The plasma concentration at which the neutralization rate equals 0.5 is given as the half-maximal neutralization concentration, and the reciprocal value is the NT$_{50}$.

**Analysis of the Mode of Action of Neutralizing Antibodies**

To screen plasma samples from elite neutralizers for their mode of action, their effect on adsorption or penetration of virions was discriminated using the dual-fluorescent HCMV strain TB40-BAC$_{KL7}$-UL32EGFP-UL100mCherry [41], including HCMV-negative plasma as a negative control. HFFs were seeded at $7.5 \times 10^5$ cells/well on a 96-well plate (µClear [black]; Greiner Bio-One). Freshly produced virus was preincubated with 10 mmol/L EGTA, 60 mmol/L PIPES, and 2 mmol/L MgCl$_2$ solution (10% sucrose, 1% fetal calf serum in PHEM buffer, and 0.5% Nonidet P40). Subsequently, the cells were stained with anti–α-tubulin monoclonal mouse antibody (Life Technologies) and AlexaFluor350-conjugated goat anti-mouse IgG F(ab')$_2$ (Life Technologies), each diluted in PHEM buffer. Five pictures of each condition were taken at 640-fold magnification with a fluorescence microscope (Axioobserver D1; Zeiss) and evaluated for green and red dot-like fluorescence signals indicative of virions. Virions with green and red fluorescence represent intact enveloped virions attached to cells, whereas virions without the red signal have penetrated cells.

Purified IgGs were analyzed by the same assay with the following modifications: HFFs were seeded at $1 \times 10^4$ cells/well. The mCherry signal was enhanced by immunofluorescence staining with rabbit anti-DsRed polyclonal antibodies (Clontech) and Cy3-conjugated goat anti-rabbit IgG F(ab')$_2$ (Jackson ImmunoResearch). Instead of tubulin staining, nuclei were counterstained with DAPI (Sigma-Aldrich). In each of 3 experiments, 6–10 pictures were randomly taken for each condition, and the numbers of particles with green and red fluorescence or green without red fluorescence were counted.

**Statistical Analysis**

Datasets were analyzed by Kruskal-Wallis 1-way analysis of variance on ranks with the Dunn post hoc test, using the build-in data analyses function of Sigmaplot. If analysis of variance indicated significant differences between groups, $P$ values were determined using unpaired 2-sided $t$ tests. Differences were considered marginally significant when $P$ values were <.05, significant when $P$ values were <.01, and highly significant when $P$ values were <.001.

**RESULTS**

**Identification of Top Neutralizers Among HCMV-Seropositive Blood Donors**

To identify routine blood donors with exceptionally high neutralizing capacities, retained plasma samples from 9000 HCMV-seropositive donors were screened for efficient inhibition of HCMV strain TB40/E infection in fibroblast cultures. Using a recently developed Gaussia luciferase–based neutralization assay, plasma samples were tested at a 1:400 dilution in comparison with a highly neutralizing preselected reference plasma to discriminate the 2.5% top-neutralizing samples [21]. If the neutralizing effect exceeded that of the reference (ie, if the ratio of the RLU for reference plasma to the RLU for test plasma was ≥1.1), the respective plasma sample was regarded as a top-neutralizing specimen (Figure 1A). A total of 236 samples (2.6%) fulfilled this criterion, indicating that the strategy of the screening approach worked as intended.

The 80 plasma samples with the highest score were then selected for a subsequent quantitative analysis. Serial dilutions of each plasma specimen were tested in an ELISA-based assay for their effect against strain TB40/E in fibroblasts, and the NT$_{50}$ for HFFs was calculated. The NT$_{50}$ values ranged between 250 and 2950. The majority of samples (64 of 80) had an NT$_{50}$ of >500, and 33 of 80 had an NT$_{50}$ of >1000 (Figure 1B). When a plasma unit (300 mL) is administered to a patient, it will be diluted about 100-fold in the interstitial fluid compartment [42]. Hence, plasma units from these top neutralizers are expected to achieve titers in vivo that are effective against HCMV, based on our in vitro assays. In summary, we successfully identified top-neutralizing plasma samples for further characterization of the breadth of their neutralizing effect in different cell types and against a panel of HCMV strains.

**Identification of Elite Neutralizers With Broad Reactivity**

Our previous work suggested that plasma with a high neutralization capacity against fibroblast infection usually has at least an equally high neutralization capacity against endothelial cell infection [21]. To test whether this assumption holds true for our 80 top neutralizers, we determined the neutralization titers of their plasma specimens against endothelial cell infection. The ratio of the NT$_{50}$ for HECs to that for HFFs was calculated as a readout for the relative efficiency in HECs versus HFFs, and the log of this ratio was plotted in the order of size for all plasma samples (Figure 2A). A ratio of the log NT$_{50}$ for HECs to the log NT$_{50}$ for HFFs of 0 indicates equal neutralization capacity in both cell types, whereas a log value >0 indicates higher efficiency against infection of endothelial cells. As expected, almost all plasma samples (77 of 80) were more potent against endothelial cell infection. This confirmed our strategy to screen with
a simplified assay only in fibroblasts and then validate the top scorers also on endothelial cells.

The next step was to identify neutralizers with broad activity against 7 HCMV strains covering most genotypes of HCMV envelope glycoproteins [21]. Plasma samples with NT50 values of >100 against all strains were considered broadly neutralizing (Figure 2B). This cutoff was chosen because clinical application of a plasma unit will result in a dilution of about 1/100 in the interstitial fluid, and the same value has been used to define broadly neutralizing plasma in the HIV field [23]. According to this definition, 58 of the 80 tested top neutralizers (71%) showed broadly neutralizing activity against all strains (Supplementary Materials).

In summary, 58 plasma specimens (0.6%) had exceptionally high and broad effective neutralizing ability and were hence designated as elite-neutralizing samples.

**Plasma Specimens From Elite Neutralizers Are More Potent Than Currently Available Anti-HCMV Immunoglobulins**

Next we tested whether plasma specimens from elite neutralizers are more effective at neutralizing HCMV than commercial hyperimmunoglobulins generated from sera from donors who were selected on the basis of HCMV antibody titers determined by ELISA. Therefore, the neutralizing capacity of 3 elite-neutralizing plasma samples were compared to that of a commercially available HCMV hyperimmunoglobulin and standard immunoglobulin. The latter was included as a baseline control.

The neutralizing capacity of all preparations was measured by the ELISA-based neutralization assay and plotted against the total IgG concentration or the CMV-specific antibody concentration. Comparison of the total IgG concentration reflects the overall neutralizing capacity of the antibodies (Figure 3A), whereas comparison regarding the virus-specific anti-HCMV IgG reflects the specific neutralizing quality of the HCMV-specific antibodies in the sample (Figure 3B). When standardized to the total IgG content, standard immunoglobulin showed the lowest neutralizing capacity, followed by hyperimmunoglobulin, and all 3 elite-neutralizing plasma specimens had higher neutralizing capacities. When standardized to anti-CMV IgG, it became apparent that the anti-CMV antibodies of standard immunoglobulins and hyperimmunoglobulins had similar neutralizing abilities. In contrast, the preselection of elite-neutralizing donors for exceptionally high neutralization titers was reflected in an approximately 10-fold higher specific quality of their anti-CMV antibodies.

**Blocking of HCMV Adsorption and Penetration by Elite-Neutralizing Plasma Specimens**

Blocking of various steps of viral entry into the host cell is the most likely mechanism used by antibodies to neutralize viral infection. Therefore, we investigated the mode of action of HCMV-neutralizing antibodies in elite-neutralizing plasma specimens with regard to inhibition of HCMV adsorption and penetration into the host cell. To investigate more precisely which of these entry steps is blocked by plasma specimens from elite neutralizers, we used a dual fluorescent HCMV assay (HCMV-TB40-BAC4-UL32EGFP-UL100mCherry), in which...
capsid-associated and envelope-associated proteins are labeled with green and red fluorescent tags [41]. This allowed to distinguish complete virus particles that have attached to the cell from virus particles that have successfully penetrated into the cytoplasm and thereby lost their envelope.

Each of the 58 elite-neutralizing plasma samples was adjusted to 10 times the NT<sub>50</sub> for HFFs and preincubated with the dual fluorescent HCMV for 2 h. The mixtures were then added to fibroblasts for 2 hours to allow adsorption and penetration of the virus. Subsequently, the plasma-treated cultures were fixed and analyzed for green and red fluorescent signals among individual virus particles and compared to cultures treated with HCMV-negative plasma. Enveloped particles displayed fluorescence in both the green and the red channels, whereas naked particles (ie, virions without an envelope) were fluorescent only in the green channel. Naked particles were assumed to have penetrated into the cytoplasm. Three different effects on HCMV particle entry could be discriminated (Figure 4). First, 42 of 58 plasma specimens showed inhibition of penetration (ie, the total particle number was unaffected, but most particles retained their envelope). Second, 2 of 58 specimens inhibited the adsorption (ie, the total number of particles was greatly reduced, but most of them succeeded to penetrate). Third, 6 of 58 specimens caused an aggregation of particles (ie, enlargement and increased intensity of fluorescence signals indicating aggregation of individual particles, which resulted in reduced infection efficiency in the immediate early antigen control staining). A combined effect was observed in 8 of 58 samples.

To confirm that these effects were actually due to the antibodies within the plasma samples, the respective blood donors were revisited, additional plasma samples were obtained, and IgGs were isolated and analyzed with the same assay. Purified IgGs from 4 donors with penetration-inhibiting specimens had a significantly reduced number of penetrated particles, as compared to IgG from an HCMV-seronegative donor (Figure 4B), whereas the IgG preparations from 2 donors with adsorption-inhibiting
specimens significantly reduced the total number of attached particles (Figure 4C). In all cases, the mode of action of the complete plasma was reflected in the activity of purified IgGs from the respective donor.

Taken together, antibodies from the elite neutralizers with the greatest neutralization capacity primarily blocked penetration of virus particles, but 2 elite neutralizers with an exceptional neutralization capacity produced antibodies that blocked HCMV at the level of virus attachment.

Neutralizing Capacities of Elite Neutralizers Remain Stable Over Several Years

Finally, we tested whether neutralization titers of elite neutralizers are stable over time. Therefore, retained plasma samples from 6 donors who had donated repeatedly for 5 years were investigated to determine neutralizing capacities against both cell types, and total anti-HCMV-IgG levels were also determined (Figure 5).

In concordance with data shown in Figure 2A, the NT50 values for HECs exceeded those for HFFs almost 10-fold, with the exception of donor 2, who had similar neutralization capacities against both cell types, and total anti-HCMV-IgG levels were also determined (Figure 5).

DISCUSSION

Using a systematic screening approach, we identified blood donors with exceptionally potent and broadly neutralizing antibodies against HCMV. Several lines of evidence suggest that anti-HCMV antibodies can in principle reduce the risk of vertical HCMV transmission during pregnancy and the extent of viremia and HCMV-associated morbidity in immunocompromised patients [13, 14, 43, 44]. At present, however, neither natural humoral immune responses nor active or passive immunization are sufficient to provide a reliable protection [9, 12, 45], and the extent to which neutralization or more-indirect mechanisms, such as antibody-dependent cellular cytotoxicity or complement activation, contribute to protection by antibodies is unclear. It was suggested that the efficacy of passive immunization could be increased by improving the quantity and/or quality of the neutralizing antibodies as compared to that of currently available immunoglobulin preparations [46–48]. A combination of 2 neutralizing monoclonal antibodies recently yielded encouraging results in kidney transplant recipients [20]. Therefore, we aimed to identify elite-neutralizing blood donors with exceptionally broad and potent antibody responses against HCMV.

The concept of elite neutralization was developed in the HIV field: although most HIV-infected individuals produce neutralizing antibodies, escape mutants often develop, and the humoral immune response hence fails to control virus replication. A minority of HIV-infected individuals, however, will develop
Figure 4. Mode of action of the elite-neutralizing plasma samples. A, The effect of all 58 plasma samples with broad and potent neutralization capability (obtained from elite neutralizers) on adsorption and penetration was investigated with a dual-fluorescent derivative of human cytomegalovirus (HCMV), TB40/E, in which the envelope glycoprotein gM is tagged with mCherry and the capsid-associated tegument protein pp150 is tagged with enhanced green fluorescent protein. Dual-labeled virus was preincubated for 2 hours with each plasma sample at a concentration reflecting 10 times the half-maximal neutralization titer. The mixture was then added to fibroblast cultures and, after 2 hours of infection, the effects of the respective plasma on adsorption and penetration were analyzed. Yellow dot-like signals represent enveloped (ie, nonpenetrated) viral particles (indicated by arrows), whereas green dot-like signals represent penetrated particles (capsids) that have already lost their envelope (indicated by open arrows). Red dots represent viral defective particles (dense bodies). Red patchy signals in the perinuclear region of the control represent gM accumulations occurring after capsid penetration. Exceptionally, larger signals were found that represent aggregation of several particles (indicated by white asterisks). Cells were counterstained for α-tubulin, which is displayed in white. Events falling in the different categories were counted and compared to values obtained with cultures infected in the absence of antibodies (control). The pie chart shows that 42 plasma samples inhibited penetration, whereas only 2 plasma samples specifically inhibited adsorption, 6 plasma samples caused aggregation, and 8 plasma samples had combined effects.

B, To test whether the effects on penetration were actually due to antibodies, purified immunoglobulin G (IgG) from 4 donors with penetration-inhibiting plasma were retested with the dual fluorescent virus. IgG from an HCMV-seronegative donor was used as reference, and the particle numbers were separately normalized to the reference values for each experiment. Each bar represents one donor, indicated as numbers on the x-axis. All 4 preparations significantly reduced the number of penetrated particles.

C, To test whether the effects on adsorption were actually due to antibodies, purified IgG from the 2 adsorption-inhibiting plasma samples were retested with the dual fluorescent virus. IgG from an HCMV-seronegative donor was used as a reference, and the particle numbers were separately normalized to the reference values for each experiment. Each bar represents 1 donor, indicated as numbers on the x-axis. Error bars represent standard errors of the mean. neg, negative. ***P < .001.
exceptional antibodies that are highly potent and broadly active against most genetic variants of HIV [23]. Such antibodies are now evaluated in clinical trials to improve antibody-based interventions for the treatment of HIV infection [49]. The situation with HCMV is similar, since most HCMV-infected individuals develop a neutralizing antibody response that does not reliably limit viral replication or transmission [9]. It is tempting to assume that—like in the HIV field—elite HCMV neutralizers with exceptionally potent and broad antibodies exist and may serve as a source to improve passive immunization.

Ideally, plasma with an elite capacity for HCMV neutralization should be highly efficient against a variety of virus strains and against both the pentamer-dependent and the trimer-dependent HCMV entry pathway. Fifty-eight of 9000 HCMV-seropositive blood donors (0.6%) had plasma specimens that fulfilled these criteria, which resembles the frequency of donors (1.7%) demonstrating elite anti-HIV neutralization capacity when similar criteria were used (ie, neutralization titers of >100 against various virus strains). These elite-neutralizing donors had remarkable qualitative features. First, their antibodies exhibit more-balanced activity against the 2 entry routes. With elite-neutralizing donors, the ratio of the NT\textsuperscript{50} for HECs to that for HFFs was about 2, whereas it is usually about 10. This could indicate that their antibodies are particularly active against the trimer-dependent route. Second, the neutralizing capacity of their anti-HCMV antibodies appears to be up to 10-fold higher than that of normal immunoglobulin and HCMV hyperimmunoglobulin. Third, 2 elite neutralizers inhibited HCMV infection at the level of virus attachment. B cells producing anti-HCMV antibodies have been successfully cloned from seropositive individuals [50], and it is therefore tempting to speculate that cloning B cells from elite neutralizers could yield particularly potent anti-HCMV antibodies. In particular, a targeted combination of penetration inhibitors and attachment inhibitors directed against gB, trimer, and pentamer may be beneficial due to additive effects.

Regarding clinical applications of plasma from elite-neutralizing donors, it is relevant that their neutralization capacities were stable over long periods. They can therefore be revisited repeatedly, either for collection of plasma specimens for direct therapeutic use or for isolation of B cells that produce antibodies with particular qualities.

In conclusion, screening of HCMV-seropositive blood donors with a 2-step selection procedure helped identify elite HCMV-neutralizers with broad and potent anti-HCMV capacities. Plasma specimens of such donors might serve as a basis for improved passive immunization strategies.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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References


4. Detection of Antibody-secreting Cells Specific for the Cytomegalovirus and Herpes Simplex Virus Surface Antigens

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Jessica Falk established and performed the „Antibody-Footprint assay“ for HCMV. The manuscript part for HCMV and corresponding visualization was done by Jessica Falk. Revision of the HCMV part of the manuscript together with Christian Sinzger and Adalbert Krawczyk
Detection of antibody-secreting cells specific for the cytomegalovirus and herpes simplex virus surface antigens

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ABSTRACT

Infections with the herpes simplex virus (HSV) and the human cytomegalovirus (HCMV) can lead to life-threatening diseases, particularly in immunosuppressed patients. Furthermore, HSV infections at birth (herpes neonatorum) can result in a disseminated disease associated with a fatal multiorgan failure. Congenital HCMV infections can result in miscarriage, serious birth defects or developmental disabilities. Antibody-based interventions with hyperimmunoglobulins showed encouraging results in clinical studies, but clearly need to be improved. The isolation of highly neutralizing monoclonal antibodies is a promising strategy to establish potent therapy options against HSV and HCMV infections.

Monoclonal antibodies are commonly isolated from hybridomas or EBV-immortalized B-cell clones. The screening procedure to identify virus-specific cells from a cell mixture is a challenging step, since most of the highly neutralizing antibodies target complex conformational epitopes on the virus surface. Conventional assays such as ELISA are based on purified viral proteins and inappropriate to display complex epitopes.

To overcome this obstacle, we have established two full-virus based methods that allow screening for cells and antibodies targeting complex conformational epitopes on viral surface antigens. The methods are suitable to detect surface antigen-specific cells from a cell mixture and may facilitate the isolation of highly neutralizing antibodies against HSV and HCMV.

1. Introduction

Neutralizing antiviral antibodies have become a potent tool for the treatment and prevention of severe viral infections during the past decades (Marasco and Sui, 2007). Polyclonal immunoglobulin G (IgG) preparations derived from immunized human donors are used against a wide range of viral infections, such as the human cytomegalovirus (HCMV), respiratory syncytial virus (RSV), hepatitis B virus (HBV), rabies and other viral infections (Both et al., 2013). However, the efficacy of such preparations is limited since virus-specific neutralizing antibodies are only a minor proportion of the total pool of polyclonal serum-derived antibodies (Marasco and Sui, 2007). The development of the hybridoma technology for the production of monoclonal antibodies by Köhler & Milstein in 1975 was a mile stone in the antibody field and led to the isolation of numerous monoclonal antibodies (mAbs) (Köhler and Milstein, 1975). From then on, numerous potent antiviral monoclonal antibodies against H5N1 influenza virus, human immunodeficiency virus (HIV), hepatitis C virus (HCV), Ebola virus, severe acute respiratory syndrome-related coronavirus (SARS-CoV) and other viruses causing dangerous infections were isolated and are currently in clinical studies or even approved for antiviral treatment (Bornholdt et al., 2016; Both et al., 2013; Caskey et al., 2016; Kwong et al., 2013; Pelegrin et al., 2015; Traggiai et al., 2004). Human monoclonal antibodies can be isolated from the B-cells of patients who have recovered from disease (e.g. SARS or Ebola) or are long-term controllers of chronic infections (e.g. HIV or herpesviruses). Several technologies are available including the immortalization of human B-cells with the Epstein-Barr virus (EBV) (Ali et al., 2015; Traggiai et al., 2004), generation of stable human hybridomas (Yu et al., 2008), direct cloning of the heavy and light antibody chains (VH and VL genes) into
phage expression libraries (Diebolder et al., 2014; Marks et al., 1991; McCafferty et al., 1990) or the generation of monoclonal antibodies from single human B-cells by single cell PCR (Tiller et al., 2008; Wardemann et al., 2003). Many of these strategies are time-consuming and laborious. In the case of working with EBV-immortalized B-cells or hybridomas, the sorting of antigen-specific donor B-cells prior to culturing can significantly facilitate the identification of rare B-cells secreting neutralizing antibodies (Kodituwakkul et al., 2003; Morris et al., 2011; Potzsch et al., 2011; Zhang et al., 2016). For this purpose, donor B-cells are presorted by flow cytometry techniques using recombinant viral glycoproteins. This strategy is highly promising if the target antigen of the neutralizing antibodies is known or if the virus incorporates only one or two surface glycoproteins as potential targets of neutralizing antibodies (e.g. Ebola) (Zhang et al., 2016). In the case of complex viruses like the human cytomegalovirus (HCMV) or the herpes simplex viruses (HSV), an enrichment of antigen-specific B-cells may be challenging, particularly if the target antigen is not exactly known. Herpesviruses use complex entry machinery consisting of numerous glycoproteins (Connolly et al., 2011; Sathiyaamoorthy et al., 2017). Four viral glycoproteins (gB, gD, gH and gL) are required and sufficient for HSV-1 and 2 entry into host cells (Agelidis and Shukla, 2015). HCMV uses two distinct pathways to enter host cells. While gB represents the fusion protein, two different glycoprotein complexes control the cell tropism of the virus: the gH/gL/gO trimer is involved in the infection of all cell types, while the gH/gL/pUL128/pUL130/pUL131A pentamer is additionally required for the infection of endothelial, epithelial and myeloid cells (Kabanova et al., 2016; Vandas and Johnson, 2012; Zhou et al., 2015). Neutralizing antibodies targeting such complexes often bind to a conformation-dependent epitope (Britt and Auger, 1985). Both cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum (PAN-Biotech, Aidenbach, Germany), 100 µg/ml gentamicin (Sigma-Aldrich), 25 µM 2-Mercaptoethanol (Sigma-Aldrich), 2% murine interleukin 6 (IL-6, 100 U/µl, PeproTech, Hamburg, Germany). Primary human foreskin fibroblasts (HFFs) were cultured in MEM supplemented with Glutamax (Life Technologies Gibco), 5% fetal bovine serum (PAN-Biotech), 0.5 ng/ml basic fibroblast growth factor (bFGF, Life Technologies Gibco) and 100 µg/ml gentamicin (Sigma-Aldrich). During infection, bFGF was omitted from HFF-medium (denoted as MEMs). Stably transfected Sp2/0-Ag14 cells secreting an HSV-1/2 specific, humanized antibody mAB hu2c were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific). For the generation of cells transiently expressing a human anti-HCMV antibody, HEK-293 T cells (highly transfectable derivative of the human primary embryonic kidney cell line 293, DSMZ) were transiently transfected (K2 Transfection System, Biontex, Munich, Germany) with two plasmids encoding the heavy and light chain of human IgG 8 J16, a neutralizing antibody directed against the HCMV pentameric complex (Macagno et al., 2010). An eGFP-encoding plasmid (pEGFP-N3, Takara, Mountain View, USA) was used to control transfection efficiency, which was around 90%. Supernatants of 8 J16-transfected cells were specifically neutralizing endothelial cell infection, as intended (not shown). HEK-293 T cells were cultured in DMEM (Life Technologies Gibco) supplemented with 10% fetal bovine serum (PAN-Biotech) and 100 µg/ml gentamicin (Sigma-Aldrich).

2.2. Virus

Recombinant, Fc-receptor (ΔE) deleted herpes simplex virus 1 (HSV-1 ΔE) was described previously (Farnsworth et al., 2003), kindly provided by Hartmut Hengel (Institute of Virology, Freiburg, Germany) and propagated and titrated on Vero cells. Virus stocks containing 2 × 10^7 TCID₅₀/ml HSV-1 ΔE were UV inactivated for 30 min (UV analysis lamp, 254 nm, Herolab, Wiesloch, Germany) and stored at −20 °C. thawed stocks were then used for coating of microtitre plates. The GFP-expressing reporter virus RV-TB40-ΔACKL7-SE-EGFP was generated as described previously, has a self-excisable BAC-cassette, and GFP expression is controlled by a shortened IE-promoter (Sampaio et al., 2017).
2.3. Antibody production and purification

For antibody production, the cells were cultured under serum-free conditions in EX-CELL Sp2/0 Serum-Free Medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific). Monoclonal antibodies (mAb) 2c (IgG2a), mAb hu2c (IgG1), H34 and IM9 were purified from serum-free cell-culture supernatants by protein A chromatography (Thermo Scientific, Worcester, MA, USA) as previously described (Krawczyk et al., 2013; Krawczyk et al., 2011) and dialyzed against phosphate-buffered saline (PBS).

2.4. Identification of HSV-specific hybridoma cells and antibodies with a virus-based ELISA assay

Microplates (96-well plates, Greiner Bio One, Kremsmünster, Austria) were coated with an UV inactivated HSV-1 ΔgE overnight at 4 °C. The immobilized virus was then fixed with 2% paraformaldehyde (PFA, Carl Roth, Karlsruhe, Germany) for ten minutes and washed three times with PBS. Non-specific binding was blocked with PBS containing 0.5% BSA (Life Technologies Gibco) for 1 h at room temperature. Subsequently, the plates were washed three times with PBS. Immobilized HSV-1 ΔgE was then incubated with different concentrations (500, 100, 50, 25, 10, 5 and 1) of murine hybridoma cells secreting the monoclonal antibody mAb 2c and SP2/0 cells secreting the humanized counterpart mAb hu2c. Both antibodies recognize a conformational epitope on the HSV-1 gE. The cells were diluted in culture medium supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, respectively. As control, either murine H34-hybridoma cells or human IM9 multiple myeloma cells were added as an unspecific control to reach a total number of 500 cells/well. The cultures were then incubated for 48 h at 37 °C. The plates were washed five times with PBS containing 0.2% Tween 20 thereby lysing the cells in order to remove cells and unbound antibodies. Bound antibodies were detected using an ultra-sensitive ABC mouse IgG staining kit (Thermo Fisher Scientific, cat. no. 32052). The kit includes a goat-anti-mouse (GAM) IgG-specific, biotin-conjugated secondary antibody. The bound secondary antibody was then incubated with avidin-horseradish peroxidase (HRP) complexes to maximize the signal. The secondary antibody in combination with the avidin-peroxidase complex was incubated for 1 h at room temperature, followed by washing three times with PBS 0.2% Tween 20. The same kit was used to detect bound immobilized mAb hu2c, but the secondary antibody was replaced by a biotin conjugated goat-anti-human (GAH) IgG-specific secondary antibody (cat. no. 109-065-008, Jackson ImmunoResearch, Cambridgeshire, UK).

TMB-ELISA substrate (Thermo Fisher Scientific) was then incubated for up to 15 min, until a characteristic colour change was detectable. The reaction was stopped with 2 M sulfuric acid (AppliChem, Darmstadt, Germany) and the signal was measured by spectrophotometry (Berthold Technologies, Bad Wildbad, Germany) at 450 nm. Cut-off was defined as a 3-fold value of the PBS control.

To determine the detection limit of this assay, serial dilutions (500–0.03 nM) of purified monomeric antibody mAb 2c or mAb hu2c were incubated on HSV-1 ΔgE coated plates for 1 h at 37 °C. Bound antibodies were detected as described above.

To determine the sensitivity of the virus-based ELISA assay, decreasing concentrations (250–0 nM) of mAb 2c or mAb hu2c were incubated on HSV-1 ΔgE coated plates and the bound antibodies were measured as described above. A murine, Friend virus-specific control antibody H34 or a human antibody IM9 not specific for HSV-1 was used to generate background values at each dilution (250–0 nM).

2.5. Identification of HCMV-specific cells with an antibody-footprint assay

2.5.1. Detection of HCMV-specific hybridoma cells

Microplates (96-well µClear high-bind black, Greiner Bio One) were coated with a goat F(ab')2 anti-mouse IgG, specific for Fcy (13 µg/ml, Jackson ImmunoResearch, West Grove, PA, USA) in 100 mM carbonate buffer (pH 9.6) overnight at 4 °C. Non-specific binding was blocked with PBS containing 5% milk powder (Sigma Aldrich) for 2 h at room temperature. The blocking solution was removed. Trypan blue (Sigma-Aldrich) was used for counting the hybridoma cells, hence excluding dead cells. Varying numbers of HCMV gH-specific H10–hybridoma cells (e.g. 500, 100, 50, 25, 10, 5 and 1) were mixed with 28–77–hybridoma cells, serving as unspecific control to reach a total number of 500 cells/well. The hybridoma cells were seeded in RPMI medium (Life Technologies Gibco) supplemented with 10% fetal bovine serum (PAN-Biotech), 100 µg/ml gentamicin (Sigma Aldrich), 25 µM 2-mercaptoethanol (Sigma-Aldrich), 2% murine interleukin 6 (IL-6, 100 U/ml, PeproTech) on the plates with the immobilized goat anti-mouse capture antibody. The cultures were then incubated overnight at 37 °C. In order to lyse the cells, the plates were washed three times with 0.2% Tween 20 in H2O. The reporter virus RV-TB40-BACΔ7-SE-EGFP was diluted in MEM5 to 5 × 10^4 IU/ml (infectious units per ml) and incubated on the plates for 2 h at 37 °C. The unbound virus was removed by three washing steps with MEM5. HFF cells were seeded on the plate in MEM5 with a density of 2.5 × 10^5 cells per well. After two days of incubation at 37 °C, foci were visualized with an Axio Observer D1 microscope (Zeiss, Oberkochen, Germany). To visualize all footprints in one well, six pictures were taken at a 40-fold magnification and merged.

2.5.2. Identification of HEK-293 T cells secreting an HCMV specific human antibody

Microplates (96-well µClear high-bind black, Greiner Bio One) were coated with a goat F(ab')2 anti-human IgG, specific for Fcy (15 µg/ml, Jackson ImmunoResearch, West Grove, 216 PA, USA) in 100 mM carbonate buffer (pH 9.6) overnight at 4 °C. Non-specific binding was blocked with PBS containing 5% milk powder (Sigma Aldrich) for 2 h at room temperature. The blocking solution was removed and HEK-293 T cells, transiently expressing an HCMV specific human IgG 8 J16, were seeded on the microplate in a serial dilution. In parallel, stably transfected Sp2/0.Ag14 cells secreting a humanized antibody mAb hu2c were seeded serving as unspecific control. Cells were incubated for two days at 37 °C. In order to lyse the cells, the plates were washed with 0.2% Tween 20 in H2O and further three times with MEM5. The reporter virus RV-TB40-BACKL7-SE-EGFP was diluted in MEM5 to 5 × 10^5 IU/ml (infectious units per ml) and incubated on the plates for 2 h at 37 °C. The unbound virus was removed by three washing steps with MEM5. HFF cells were seeded on the plate in MEM5 with a density of 2.5 × 10^5 cells per well. After two days of incubation at 37 °C, foci were visualized with an Axio Observer D1 microscope (Zeiss, Oberkochen, Germany). To visualize all footprints in one well, six pictures were taken at a 40-fold magnification and merged.

3. Results

Identification of antigen-specific antibody-secreting cells, such as B-cells, hybridomas or cells transfected with plasmids encoding IgG-genes, is a crucial step during the process of isolating highly neutralizing antibodies. To facilitate this step and thereby promote the generation of novel broadly neutralizing antibodies against HSV and HCMV, we established two screening approaches to identify virus-specific antibody-secreting cells. Since the entry of HSV and HCMV is a highly complex process involving several viral surface proteins, we used intact virus particles for the screening procedure. This strategy allows identifying antibodies directed against the complex entry machinery including miscellaneous conformational epitopes.

3.1. Virus-based ELISA for identification of HSV-specific cells

3.1.1. General description

For the identification of HSV-specific cells, we established an ELISA
that is based on the detection of HSV-specific antibodies bound to immobilized virus (Fig. 1). The assay is designed for the screening of antibody-secreting cells or cell culture supernatants for HSV-specific antibodies. Microtiter plates are coated with HSV-1 ΔgE lacking the HSV-Fc-receptor (gE/gI complex) to minimize unspecific binding of antibodies (Fig. 1A). The binding of the IgG-Fc domain to the HSV-Fc-receptor is species-specific. In contrast to murine antibodies, human IgGs bind to the HSV-1 Fc-receptor (Sprague et al., 2006). To exclude unspecific binding when working with human antibody-secreting cells or antibodies in subsequent studies, we decided to establish this assay with the HSV-1 ΔgE mutant. The immobilized virus is then fixed and overlaid with murine human antibody-secreting cells. The cells are incubated for 48 h with the immobilized virus (Fig. 1B). Subsequently, antibody-secreting cells and HSV-unspecific antibodies are removed (Fig. 1C). Bound HSV-specific antibodies can be detected with a peroxidase conjugated secondary antibody (Fig. 1D). Cell cultures positive for virus-specific antibodies can subsequently be diluted to single cell level, expanded, retested for specific antibodies and selected for the production of monoclonal antibodies.

3.1.2. Detection of cells secreting HSV-specific antibodies with a virus-based ELISA assay

The virus-based ELISA assay is suitable for identifying cells (hybridoma cells, B-cell clones or transfected cells) secreting HSV-specific antibodies directed against viral surface antigens. The method was established for the detection of cells secreting murine and human antibodies. As a model system for cells secreting murine or human antibodies, we used a murine hybridoma cell line 2c and a stably transfected Sp2/0-Ag14 cell line producing the humanized antibody mAb hu2c. Both antibodies are specific for a conformational epitope on the glycoprotein B of HSV-1/2 (Eis-Hubinger et al., 1993; Krawczyk et al., 2013; Krawczyk et al., 2011). Microtiter plates are coated with HSV-1 ΔgE. Immobilized HSV-1 ΔgE was then incubated with distinct numbers of either 2c-hybridoma cells or Sp2/0-Ag14 cells (500, 100, 50, 25, 10, 5 and 1) secreting murine or humanized HSV-1 specific antibodies. The cells were mixed with the respective number of murine, Friend-Virus-specific H34 hybridoma cells or human IM9 cells secreting a monoclonal antibody not specific for HSV-1 as an unspecific control to reach an equal number of 500 cells per well. H34 or IM9 cells alone served as a background control. The cut-off was defined as the twofold value of the unspecific background measured for 500 H34 or IM9 cells, respectively.

The assay proved to be reliable to detect five murine hybridomas (Fig. 2A) or ten cells secreting a humanized antibody specific for HSV-1 (Fig. 2C) from a total number of 500 cells. The test was performed three times in triplicates with similar results, which demonstrated the reproducibility of the assay. Only a slight background signal was observed when using H34 or IM9 cells alone. We conclude that the screening procedure can be performed as a high throughput assay to identify HSV-specific antibody-secreting cells.

3.1.3. Sensitivity of the HSV-1 ΔgE based ELISA

To determine the detection limit and sensitivity of the virus-based ELISA, we incubated various concentrations (500–0 nM) of the murine mAb 2c or the humanized mAb hu2c on immobilized HSV-1 ΔgE. The binding of these HSV-specific antibodies to the immobilized virus was quantified by the activity of the peroxidase-conjugated secondary antibodies. The cut-off was defined as a threefold value of the PBS signal. We found that bound mAb 2c was detectable at concentrations ≥0.98 nM (Fig. 2B) and mAb hu2c at concentrations ≥3.91 nM (Fig. 2D). However, unspecific binding of antibodies that are not directed against HSV might lead to false positive results, particularly at the low concentration range.

To investigate the impact of unspecific antibody binding, we repeated the measurements and compared the binding properties of mAb 2c and mAb hu2c to those of the murine Friend virus-specific antibody H34 or the human antibody IM9 that is not specific for HSV-1. The detection limit was defined as a threefold value of the PBS control. HSV-1 specific binding could be detected at concentrations ≥1.95 nM mAb 2c (Fig. 3A) or mAb hu2c (Fig. 3B).

A slightly background signal was observed only at a concentrations of 250 nM H34 or IM9. However, it was considerably lower than the mAb 2c or mAb hu2c signal at this concentration. These data demonstrate that the virus-based ELISA is a reliable method to detect cells secreting murine or human/humanized antibodies specific to HSV-1 surface antigens, including those recognizing conformational epitopes. The method is also suitable for detecting antibodies in cell culture supernatants.

3.2. GFP reporter virus-based assay for the detection of HCMV-specific B-cells

3.2.1. General description

To identify novel broadly neutralizing monoclonal antibodies against HCMV, we have established a HCMV-GFP-reporter virus-based assay. This assay was designed to identify cells secreting antibodies
Fig. 2. Identification of cells secreting HSV-specific antibodies. Microtiter plates were coated with HSV-1 ΔgE. Immobilized virus was incubated with various numbers (500–0) of cells secreting HSV-1 specific antibodies. (A) Murine 2c hybridoma cells secreting an HSV-1/2 gB-specific antibody were mixed with H34 hybridoma cells secreting a Friend virus-specific murine antibody. (C) Stably transfected Sp2/0-Ag14 cells (500–0) secreting an HSV-1/2 gB-specific, humanized antibody mAb hu2c were mixed with IM9 cells, which secreted a human antibody not specific for HSV-1/2. The total cell number was 500 cells/well. H34 or IM9 cells alone served as background control. After 48 h of incubation, the cells were lysed and virus-bound antibodies were detected with a peroxidase conjugated secondary antibody specific for murine (A) or human (C) IgG-Fc-fragment. (B) Serial dilutions of purified mouse antibody mAb 2c or (D) the humanized counterpart mAb hu2c were incubated on immobilized HSV-1 ΔgE. Bound antibodies were detected as described above. The experiments were performed as triplicates. The detection limit is given as a threefold value of the PBS signal (red line). Values are given as the averages of triplicates. Error bars indicate the standard deviation of the mean (SDM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Sensitivity of the HSV-1 ΔgE based ELISA. Decreasing concentrations (250–0 nM) of purified HSV-1/2 gB specific murine antibody mAb 2c (A) or humanized antibody mAb hu2c (B) were incubated on immobilized HSV-1 ΔgE. A murine, Friend virus-specific control antibody H34 (A) or a human antibody IM9 that is not specific for HSV-1 (B) was used to generate background values at each dilution (250–0 nM). The experiments were performed in triplicates. The detection limit is given as a threefold value of the PBS signal (red line). Error bars indicate the standard deviation of the mean (SDM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
detected by porter virus was added. After 2 h, unbound reporter virus was removed and HFF indicator cells were seeded. After 48 h, HCMV-infected GFP-producing HFFs were infected with the HCMV reporter virus. The virus exclusively binds to cell footprints if the captured antibodies recognize an HCMV surface antigen. (D) Subsequently, HFF indicator cells are seeded onto the microtiter-plates, thereby allowing infection at the positions of bound reporter virus. Green fluorescence indicates the presence of HCMV-specific cells in the cell culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.2. GFP-reporter virus-based detection of HCMV-specific hybridoma cells

The assay was established using the murine hybridoma clone H10 secreting antibodies specific to the HCMV envelope glycoprotein H (gH) (Falk et al., 2016). In a first series of three experiments, we tested whether our concept of detecting HCMV-specific antibody footprints is valid and footprints of infected cells only form if the seeded hybridoma cells were specific for HCMV surface proteins.

Microtiter plates were coated with a GAM-Fcy-specific capture antibody. Hybridoma cells were then seeded at a density of 500 cells per well and incubated overnight. The hybridoma cells are expected to secrete antibodies that will be bound by the capture antibodies, thus leaving an antibody-“footprint” at the position where they sedimented. HCMV-gH-specific hybridoma cells (clone H10) served as a positive control as the respective antibodies can bind to the surface of virions. Hybridoma cells of clone 28–77 served as a background control as they release antibodies directed against an HCMV tegument protein (pp65), which is not accessible at the surface of virions. In addition, a mixture of both hybridoma cells was applied, seeding 10 cells of clone H10 together with 490 cells of clone 28–77 per well. After the overnight incubation, the hybridoma cells were removed by detergent lysis, and the captured antibodies were overlaid with the reporter virus RV-TB40-BACgly-se-EGFP for 2 h. The virus is expected to bind exclusively to the captured hybridoma-“footprints” of clone H10. In contrast, antibodies of clone 28–77 are expected not to bind to the surface of virions and therefore not cause infected foci in the indicator cell layer. HFF indicator cells were then seeded onto the plate under the assumption that only those cells would be infected that lie at the “footprint”-bound virions. Two days later, GFP-producing infected cells were visualized by fluorescence microscopy (Fig. 5). As expected, foci of infected cells were not detected in wells where only cells of clone 28–77 were seeded, indicating that nonspecific focal binding of reporter virions did not occur. Under this condition, only very few dispersed GFP-positive cells could be detected (Fig. 5C). In contrast, the seeding of hybridoma clone H10 always resulted in the formation of infectious foci. When 500 cells of clone H10 were seeded, numerous foci were visible that could not be distinguished from each other (Fig. 5A). When 10 cells of clone H10 were seeded together with an excess of clone 28–77, foci of infected

Fig. 5. Detection of HCMV-specific hybridoma cell footprints by fluorescence microscopy. Microtiter plates were coated with a goat anti-mouse Fcy-specific capture antibody. (A) Hybridoma cells (clone H10) specific for the HCMV surface protein gH were seeded at a density of 500 cells per well. (B) H10 cells were seeded at a density of 10 cells per well together with 490 hybridoma cells of clone 28–77, which is specific for the HCMV tegument protein pp65. (C) Wells seeded with 500 cells per well of clone 28–77 served as a negative control. After 24 h of incubation, hybridoma cells were removed by lysis and medium containing the HCMV-BACgly-se-EGFP reporter virus was added. After 2 h, unbound reporter virus was removed and HFF indicator cells were seeded. After 48 h, HCMV-infected GFP-producing HFFs were detected by fluorescence microscopy. Six images were compiled to visualize the whole well. To facilitate the evaluation, photos are shown in an inverted mode; i.e. dark dots represent GFP-expressing cells. Each focus of GFP-expressing cells is assumed to represent one hybridoma cell footprint.
on the surface of HCMV. As control, stably transfected Sp2/0-Ag14 cells were found with the background control, most probably due to individual virions remaining on the plate after the washing procedure.

The antibody is directed against the pentameric glycoprotein complex suitable for the detection of human cells secreting HCMV specific antibody.

HCMV envelope protein reliably induced the formation of infected foci at sites where H10 cells had been sedimented before (Fig. 5B).

Taken together, hybridoma cells secreting antibodies against an HCMV envelope protein reliably induced the formation of infected foci within the indicator cell layer, whereas only few dispersed infected cells were found with the background control, most probably due to individual virions remaining on the plate after the washing procedure.

3.2.4. Sensitivity of the GFP-reporter virus based detection of HCMV-specific hybridoma cells

In order to demonstrate that the antibody-footprint assay is also suitable for the detection of human cells secreting HCMV specific human IgGs, we used transiently transfected HEK-293 T cells expressing an HCMV specific human antibody IgG 8 J16 (Macagno et al., 2010). This antibody is directed against the pentameric glycoprotein complex on the surface of HCMV. As control, stably transfected Sp2/0-Ag14 cells secreting a HSV-1 gB-specific humanized antibody mAb hu2c were used. In wells containing 500 of HEK-293 T cells secreting IgG 8 J16 numerous foci could be found, but could not be distinguished from each other (Fig. 6A). In wells containing 50 HEK-293 T cells secreting IgG 8 J16, the foci of infected cells were distinguishable (Fig. 6B). As expected, the foci of infected cells were not detected in wells where only mAb hu2c-secreting cells were seeded, indicating that there was no unspecific binding of the reporter virus. Under this condition, only a few dispersed GFP-positive cells could be detected (Fig. 6C). These results clearly demonstrate that the method presented here is suitable for the detection of cells secreting human IgGs specific for complex epitopes on HCMV surface.

4. Discussion

In the present study we have established two distinct methods for detecting cells secreting antibodies specific to viral surface-antigens of HSV and HCMV.

Infections with HSV or HCMV may lead to severe or even life-threatening diseases in immunocompromised patients or when acquired at birth (HSV) or during pregnancy (HCMV) (Berrington et al., 2009; Bhat et al., 2015; Boppana et al., 2013; Thompson and Whitley, 2011). Antivirals are available but may lead to the development of resistance (Minces et al., 2014; Morfin and Thouvenot, 2003) or are contra-indicated for the treatment during pregnancy due to potential teratogenic side effects (Kimberlin, 2001). Antibody-based strategies revealed to be highly promising to fight viral infections. Numerous potent neutralizing antibodies against HSV, SARS-CoV, Ebola virus and other viral pathogens have been isolated within the past years (Bornholdt et al., 2016; Caskey et al., 2016; Traggiai et al., 2004). Preclinical and early phase clinical trials with hyperimmunoglobulins or monoclonal antibodies targeting HSV or HCMV also have shown promising results (Krawczyk et al., 2013; Masi et al., 1995; Nigro et al., 2005; Revello et al., 2014). However, the identification of novel, highly neutralizing antibodies against these viruses would be of great benefit to significantly improve the antiviral treatment.

Commonly, monoclonal antibodies are derived from EBV-immortalized human B-cells collected from seropositive patients recovered from infection or from immunized mice (Marasco and Sui, 2007). Therefore, the screening and selection process is a crucial step to identify antigen-specific B-cells from the whole B-cell repertoire of seropositive humans or immunized mice in a limited time, particularly when looking for B-cells secreting antibodies targeting complex discontinuous epitopes on viral surface antigens. Functional assays such as a microneutralization assay are useful and contribute to the isolation of potent neutralizing antibodies against HCMV (Macagno et al., 2010). Alternatively to performing neutralization assays, which may require high amounts of purified antibodies, B-cells or cell-culture supernatants can be screened for antigen binding. Conventional ELISA assays are well established for routine diagnostics and usually based on purified viral antigens (Liermann et al., 2014). High throughput screening techniques based on antigen microarrays were developed to facilitate the screening process (De Masi et al., 2005; Tickle et al., 2015). These methods allow for the simultaneous screening of B-cells for antibody specificity to one particular antigen or to several antigens. However, since these assays are based on purified recombinant peptides or proteins, they are inadequate to detect neutralizing antibodies targeting complex surface-antigen epitopes.

With respect to identifying novel highly neutralizing antibodies against HSV and HCMV, we established two screening methods based on full viruses, which allow for the detection of antibodies targeting the complex entry machinery of these viruses including miscellaneous conformational epitopes. Notably, prior studies reported that highly potent antibodies against HSV or HCMV bind to such complex
were seeded and cultured for 48 h to allow the onset of infection. The total number of GFP-positive footprints/well was counted by fluorescence microscopy. Data are given as means of two independent experiments, each performed in triplicates. Error bars represent the standard error of the mean.

discontinuous epitopes on one or more than one viral protein (Cairns et al., 2014; Krawczyk et al., 2013; Macagno et al., 2010). Accordingly, Dengue virus (DENV) immune serum depletion studies have shown that the antibodies responsible for serum neutralizing activity following primary DENV infection target complex epitopes that exist in a correct conformational state only on the virion particle, but not on recombinant soluble forms of the viral surface antigen E (Smith et al., 2014). These data indicate that it might be of great benefit to work with full viruses when screening B-cell clones for neutralizing antibodies.

When working with herpesviruses such as HSV or HCMV, one should keep in mind that these viruses can bind antibodies via viral Fc-receptors and immunoglobulins is species-specific. IgGs from humans exhibit stronger binding to the HSV-FcR and HCMV-FcR than mouse IgG (Antonsson and Johansson, 2001; Johansson et al., 1985). The Fc-related unspecific binding can be eliminated by using an Fc-receptor-lacking virus or by blocking the viral Fc-receptors with human Fc-fragments or sera from seronegative donors. The HSV-1 Fc-receptor consisting of the glycoprotein dimer gE/gI is well characterized, and a gE-deletion mutant lacking the viral Fc-receptor is available (Farnsworth et al., 2003).

In the present study we worked with the HSV-1 gE mutant to exclude unspecific binding related to the HSV-1 Fc-receptor. There was no significant unspecific binding observed when using a human antibody not specific for HSV-1, indicating that the assay is reliable for detecting virus-specific antibodies in particular. Since there are a number of HCMV proteins described that can bind the human IgG-Fc-domain, and no appropriate deletion mutant was available, we decided to use an HCMV-GFP reporter virus. Although we could not observe any background related to HCMV-receptor binding of HCMV-unspecific antibodies in our study, such binding can easily be inhibited by pre-incubation of the reporter virus with human Fc-fragments as previously described (Antonsson and Johansson, 2001) when indicated. A critical point for identifying HSV or HCMV specific antibodies with the assays described here might be the antibody affinity. Cells secreting low-affinity HSV or HCMV specific may induce only weak signals and thus remain undetected. However, we know from the HIV, HSV or influenza field, that the best neutralizing antibodies bind the target antigens with a high affinity, at least at nanomolar range (see e.g. (Mascola and Haynes, 2013)). The assays described here were developed to support the identification of novel, highly neutralizing antibodies that can be further developed for clinical use. In line with prior studies, we expect that such antibodies will bind the target antigens with a high affinity. The affinity of the HSV-1 specific mAbs 2c and hu2c used in this study are at nanomolar range (Krawczyk et al., 2013). The affinity of the HCMV-specific IgG 8 J16 could not yet be determined, but based on a prior study we estimate that it is also in the nanomolar range (Macagno et al., 2010). Therefore, we conclude that the methods described in the manuscript are suitable for the detection of high-affinity antibodies targeting HSV or HCMV.

In summary, the whole virus-based screening assays described in this study were highly sensitive and allow the detection of between one and ten cells secreting murine or human/humanized antibodies specific to HSV-1 or HCMV from a cell mixture. After a positive selection of high-affinity binders, the newly identified antibodies need to be further characterized for poten virus neutralization to select potential candidates for clinical use. These methods described here may facilitate the screening procedure of B-cells specific for HSV and HCMV surface antigens, including those targeting highly complex epitope structures, and contribute towards identifying novel potent antibodies against HSV and HCMV.

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Microtiter plates were coated with a goat anti-human Fcy-specific capture antibody. (A) HEK-293 T cells, transiently transfected with plasmids coding for the heavy and light chain of IgG 8 J16, a human IgG specific for an HCMV glycoprotein complex, were seeded at a density of 500 cells and (B) at a density of around 15 cells per well. As control, 1000 Sp2/0-Ag14 cells secreting an HSV-1/2 specific, humanized antibody which was mAb hu2c seeded. After 24 h, cells were removed by lysis and medium containing the HCMV-BACKL7-eGFP reporter virus was added. After 2 h, unbound reporter virus was removed and HFF indicator cells were seeded on the microtiter plates. The total cell number was 500 cells/well. The cells were removed after 24 h of incubation and captured antibodies were overlaid with medium containing the HCMV-BACK7-eGFP reporter virus. After 2 h, unbound reporter virus was removed and HFF indicator cells

Fig. 7. Sensitivity of the HCMV-GFP reporter virus-based footprint assay. (A) Microtiter plates were coated with a goat anti-mouse-Fcγ-specific capture antibody. Varying numbers (50, 25, 10, 5 and 1) of clone H10 hybridoma cells specific for the HCMV surface antigen gH were mixed with HCMV surface antigen-unspecific hybridoma cells 28–77 and seeded on the microtiter plates. The total cell number was 500 cells/well. The cells were removed after 24 h of incubation and captured antibodies were overlaid with medium containing the HCMV-BACK7-eGFP reporter virus. After 2 h, unbound reporter virus was removed and HFF indicator cells

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5. Large Scale Screening of HCMV-seropositive Blood Donors Indicates that HCMV Effectively Escapes from Antibodies by Cell-associated Spread

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Jessica Falk cloned the Merlin_pAL1502-GLuc reporter virus and established the corresponding assays. High-throughput screening of plasma samples was performed together with Marina Winkelmann. All further experiments were performed by Jessica Falk. Evaluation of data, visualization and conceptualization of the first manuscript draft was done by Jessica Falk. Reviewing and editing of manuscript by Christian Sinzger.
Large-Scale Screening of HCMV-Seropositive Blood Donors Indicates that HCMV Effectively Escapes from Antibodies by Cell-Associated Spread

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Abstract: Immunoglobulins are only moderately effective for the treatment of human cytomegalovirus (HCMV) infections, possibly due to ineffectiveness against cell-associated virus spread. To overcome this limitation, we aimed to identify individuals with exceptional antibodies in their plasma that can efficiently block the cell-associated spread of HCMV. A Gaussia luciferase-secreting mutant of the cell-associated HCMV strain Merlin was generated, and luciferase activity evaluated as a readout for the extent of cell-associated focal spread. This reporter virus-based assay was then applied to screen plasma samples from 8400 HCMV-seropositive individuals for their inhibitory effect, including direct-acting antiviral drugs as positive controls. None of the plasmas reduced virus spread to the level of these controls. Even the top-scoring samples that partially reduced luciferase activity in the screening assay failed to inhibit focal growth when reevaluated with a more accurate, immunofluorescence-based assay. Selected sera with high neutralizing capacity against free viruses were analyzed separately, and none of them prevented the focal spread of three recent clinical HCMV isolates nor reduced the number of particles transmitted, as demonstrated with a fluorescent Merlin mutant. We concluded that donors with cell-to-cell-spread-inhibiting plasma are nonexistent or extremely rare, emphasizing cell-associated spread as a highly efficient immune escape mechanism of HCMV.

Keywords: cytomegalovirus; antibodies; cell-associated spread; immune evasion

1. Introduction

Human cytomegalovirus (HCMV) is a herpesvirus that is ubiquitously distributed in the human population. Whereas primary infection in immunocompetent individuals usually causes only mild symptoms or mononucleosis-like disease, immunocompromised individuals (e.g., AIDS patients or transplant recipients) can suffer from severe disease following HCMV primary infection or reactivation. Solid-organ or hematopoietic-stem-cell transplant recipients are at high risk of HCMV
infection, which can entail organ manifestations, but also indirect complications, including graft failure, graft-versus-host disease, and increased susceptibility to other infections [1–4]. Due to intrauterine infection, HCMV is also a cause of disabilities in children, often manifested as hearing loss or other neurological impairments [5]. The incidence of congenital HCMV infections varies from 0.6–0.7% in industrialized countries to 1–5% in developing countries [6], and leads to clinical findings at birth in 10–15% of cases [7,8].

Effective direct-acting polymerase inhibitors (i.e., ganciclovir, foscarnet, and cidofovir) are available, but their administration is limited due to myelotoxic or nephrotoxic side effects and the development of resistance [9–12]. Recently, a terminase inhibitor (letermovir) has been approved that appears to be less toxic, but resistance can occur [13]. For this reason, passive immunization is still a desirable alternative. Reports on the efficacy of HCMV hyperimmunoglobulins in pregnant women with primary HCMV infection [14–17] and in transplant recipients [18–20] are controversial. Interestingly, a combination of two neutralizing anti-HCMV antibodies was recently reported to reduce the frequency of HCMV viremia [21,22], and vaccination with recombinant glycoprotein B of HCMV provided partial protection that was correlated with antibody responses, although the mode of action of protective antibodies remains unclear [23–25].

The difficulties of establishing a highly effective antibody-based treatment regimen for HCMV infections may in part be due to variations in antibody sensitivity depending on the transmission mode, target-cell type, and HCMV strain. Recent clinical isolates are typically restricted to cell-associated spread in cell culture [26,27]. Subsequent passaging of clinical isolates in fibroblast cultures after isolation rapidly selects for mutations in two viral gene regions (RL13 and UL128L) that lead to the release of cell-free infectivity and restricted cell tropism [28,29]. Using passaged strains, anti-HCMV antibodies can partially reduce virus spread in endo- or epithelial cells, but are less effective against cell-associated spread in fibroblasts [30–35]. However when the cell-associated phenotype of clinical strains was recapitulated in vitro, virus spread was almost completely resistant to neutralizing antibodies; minor inhibition was only observed at very high antibody concentrations, and, as with passaged strains, it was more apparent against the virus in epithelial cells than in fibroblasts [36].

In contrast to cell-associated spread, cell-free virus can be neutralized by serum or plasma from HCMV-seropositive individuals. However, neutralization capacities vary greatly between plasma donors, and efficiency again depends on target-cell type, whereby, commonly, the infection of endo- or epithelial cells can be more effectively neutralized when compared with fibroblasts [37,38]. The exact role of various spreading modes and target-cell types in vivo is not known. Both transmission modes, however, appear to contribute in certain situations, as infectivity is almost exclusively cell-associated in the circulation [39–41], but often cell-free in body excretions like breast milk or urine [42,43]. It is tempting to assume that antibody preparations that can not only prevent cell-free infection of endo- and epithelial cells, but are also highly effective against cell-associated focal spread in stromal cells, could substantially improve antibody-based strategies against HCMV.

In the field of neutralizing antibodies, the identification of elite donors with exceptionally high and broad neutralization capacities has yielded significant improvement of antibody preparations [44–47]. We were interested to test whether this concept can also be transferred to identify blood donors whose plasma can, as an exception to the rule, effectively inhibit the cell-associated spread of HCMV. Therefore, we generated a reporter virus that allowed high throughput screening for cell-to-cell spread-inhibiting antibodies, and applied it to a large human blood-donor population for which neutralization capacities against cell-free HCMV infection were already known from a previous study [37].

2. Materials and Methods

2.1. Cell, Plasma, Serum, and HCMV Clinical Isolates

For propagation, human foreskin fibroblasts (HFF) were kept in “growth medium” containing minimal essential medium with 5% fetal bovine serum (PAN Biotech, Aidenbach, Germany),
GlutaMAX (Life Technologies, Carlsbad, CA, USA), 100 µg/mL gentamicin, and basic fibroblast growth factor (bFGF; Life Technologies, 0.5 ng/mL). Human fetal foreskin fibroblasts (HFFF-tet) cells were immortalized with hTERT and expressed the Tet-repressor [28]. For propagation, HFFF-tet cells were cultured in “growth medium”. During experiments, both HFFs and HFFF-tet cells were kept in “growth medium” without bFGF (MEM5). Conditionally immortalized human endothelial cells (HEC-LTTs) [48,49] were cultured in endothelial cell-growth medium (Endothelial Cell Growth Medium Kit, PromoCell, Heidelberg, Germany) supplemented with 2 μg/mL doxycycline (AppliChem, Darmstadt, Germany). During infection experiments, heparin and doxycycline were omitted from the medium. Cell-culture microplates were coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) prior to seeding of cells.

The German Red Cross Blood Transfusion Service, Baden-Württemberg-Hessen, provided ethylenediaminetetraacetic acid (EDTA)-plasma samples from their HCMV-seropositive blood donors. The Ethical Committee of the University of Ulm proved the project design resulting in vote No. 53/14. Initial tests were performed on plasma samples routinely available from blood donors. Plasma had to be recalcified prior to use in the screening because clotting factors within plasma interfered with exact pipetting. Recalcification was performed by a tenfold dilution of plasma in MEM5, followed by incubation at 37 °C for at least 2 h. Plasma samples were then stored at 4 °C until the day of the experiment. Individuals identified as highly and broadly neutralizing (“elite”) donors [37] were asked to donate serum samples.

Cell-free virus preparations of HCMV strain TB40-BAC4 [50] were used to test the neutralization capacity of sera. Recent clinical HCMV isolates were provided by the diagnostic laboratory of the Institute of Virology in Ulm. They originated from routine testing of throat swabs from patients of the Ulm University Medical Center. Sample material was applied to HFFs, and HCMV-positive cultures were then incubated for several weeks until they showed at least 50% cytopathic effect. Infected cells were then aliquoted and frozen at −80 °C, and the cell association of the HCMV isolates was tested by transferring culture supernatants onto adherent HFFs and immunofluorescence staining for viral immediate-early (IE) antigens one day after inoculation. Isolates were only used further if they were negative in this assay.

2.2. Generation of a Luciferase Reporter Virus Based on HCMV Strain Merlin

A Gaussia luciferase (GLuc) expression cassette was inserted in strain Merlin-pAL1502, downstream of the US34A gene. Merlin-pAL1502 is a bacterial artificial chromosome (BAC)-cloned HCMV strain that is a derivative of the repaired Merlin-BAC pAL1128 [28,51]. It has tet-operator sequences in front of the RL13 and UL128 genes. The Gaussia luciferase expression cassette consists of the HCMV immediate-early promoter, the Gaussia luciferase open reading frame, and a BGH (bovine growth hormone) polyA signal.

To insert the US34A flanking sequences, a PCR (polymerase chain reaction) product was amplified from the universal transfer construct pDrive-(IE-GLuc-PolyA)-Kan [52] with primers: 5′-CATATTATGGTGTCCTGTTTTTTCATTTTTTGGATGATTTGCTGCATAAAGCGGTTGATTA ATATGAGAATACG3′ and 5′-GACACGGGTTTTGTTAGGATAACAAAACTGCGTATCTGGATAT ATTTCATCCCAACATCGATGCAATTCCTACTTTA-3′ and electroporated into the recombination-activated Escherichia coli (E. coli) strain GS1783 [53] harboring Merlin-pAL1502. Following kanamycin selection, the marker cassette was removed from the BAC by an intrabacterial I-SceI digest and a Red-mediated recombination step. The integrity of the recombinant Merlin-pAL1502-GLuc was verified by restriction fragment length analysis (RFLA) and sequencing of the recombined regions. Sequence analysis revealed a nucleotide substitution (G instead of T) upstream of the luciferase-coding sequence, which did not disable expression of a functional luciferase. To obtain infectious virus particles, BAC DNA was isolated using the NucleoBond Xtra Midi kit (Macherey-Nagel, Düren, Germany) and transfected into HFFs by a calcium phosphate-based method (MBS Transfection Kit, Agilent, Waldbronn, Germany). Since producer cell cultures contain high levels of luciferase that
might interfere with downstream experiments, reporter-virus preparations were purified as previously described [52].

2.3. Generation of Dual-Labeled HCMV Strain Merlin-pAL1502-UL32EGFP-UL100mCherry

In a first step, the EGFP template cassette was amplified from plasmid pEP-EGFpin (Addgene plasmid #60961, Cambridge, MA, USA) with primers 5′-CCGTGCAGACATCTCCAAAGATCGAGGATACCAAGGAGGAAATGGTGAGCAAGGGCGAGGAGCT-3′ and 5′-CAGTATCTCTTTGTTTCTATGTTATATTGATG-3′ and inserted into Merlin-pAL1502 by seamless mutagenesis as described above. After removal of the selection marker, an mCherry-containing recombination fragment was generated from plasmid pEP-mCherry-in [33] with primers 5′-CCCTGCGTCTCTACTATACGTCGTTAGTTGTGTTTCTTAATTACTTGTACAGCTCGTCCATGC-3′ and 5′-CACTATCCGATGATTTCATTAAAAAGATGTCGCGTCTACGTTTTGAAGGCTACATGTGCCTCAGCAGTTAGTCAGGCGAGGAGCT-3′ and inserted into Merlin-pAL1502 by seamless mutagenesis as described above. The final Merlin-pAL1502-UL32EGFP-UL100mCherry genome was verified by RFLA and sequencing, which revealed a single amino acid deletion (R54) in the UL100 coding sequence as compared to the parental strain due to mCherry insertion. Infectious virus was generated by transfection into HFFF-tet cells as described above.

2.4. Detection of Viral Antigen via Immunofluorescence

Following fixation with 80% acetone, the cells were incubated with antibody E13 (Argene Biosoft, Verniolle, France) to detect IE antigens 1 and 2 (pUL122/123) [54]. Cy3-conjugated goat polyclonal antimouse F(ab′)2 antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary antibody. The nuclei of cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich). Visualization was done by fluorescence microscopy with an Axio Observer D1 microscope (Zeiss, Oberkochen, Germany).

2.5. Gaussia Luciferase-Based Screening of Plasma Samples for Inhibition of Cell-Associated Spread

HFFs were seeded on 96-well plates (Cellstar, Greiner Bio-One, Frickenhausen, Germany) at a density of 1.5 × 10^4 cells per well. Two days after seeding, cultures were infected with Merlin-pAL1502-GLuc using 0.002 focus-forming units (FFU) per cell. One day after infection (1 d p.i.), supernatants were replaced with recalculated plasma samples at a 50-fold dilution in MEM5. The direct-acting antivirals ganciclovir (Cymeven, 800 µM, Roche, Basel, Switzerland) and BDCRB (2-Bromo-5,6-dichloro-1-β-D-ribofuranosyl benzimidazole, kindly provided by Jens von Einem, 0.04 mM) were included as positive controls. From previous experiments, we knew that common seropositive plasma samples do not block the cell-associated spread of HCMV and resemble seronegative plasma in this assay. Therefore, we did not include seronegative controls, but rather searched for outstanding plasma from seropositive donors that would significantly deviate from the majority of the others. After 4 days (i.e., 5 d p.i.) supernatants were replaced with fresh MEM5. After further overnight incubation (i.e., 6 d p.i.), the GLuc activity in 20 µL supernatant of each well was measured as relative light units (RLU) in a multilabel microplate reader (Hidex Chameleon, Turku, Finland). As the intention of this screening was to identify plasma samples with outstanding inhibitory capacity, RLU values of individual samples were compared with the geometric mean of all plasma samples on the plate and reported as “relative luciferase activities” (RLU_sample / RLU_mean). Plates were only evaluated if direct-acting antivirals had relative luciferase activities below 0.3. Plasma samples that repeatedly failed these controls were excluded. Those plasma samples showing the strongest reduction of GLuc activity were considered for evaluation via immunofluorescence staining of viral antigens.
2.6. Focus-Expansion Assays with Clinical HCMV Isolates

Frozen stocks of HFFs, which were infected with clinical HCMV isolates and stored at low passages, were thawed, washed, and cocultured with an excess of uninfected HFFs or HEC-LTTs in 96-well microplates (Cellstar, Greiner Bio-One). This resulted in 50–100 infected cells per well surrounded by 15,000 uninfected indicator cells. Directly after seeding, highly neutralizing sera from previously identified “elite” donors [37] were added to the coculture at a final dilution of 1/50. The direct-acting antiviral ganciclovir (GCV, 800 µM) or serum from an HCMV-seronegative donor (dilution 1/50) was used as positive or negative control, respectively. After 6 days of cocultivation, cells were fixed with 80% acetone, and IE antigen was detected via immunofluorescence staining. It is noteworthy that plasma samples repeatedly had cytotoxic effects on HEC-LTT cultures during the 6 days of incubation, and, therefore, we had to use serum instead of plasma.

To confirm the capacity of the tested sera to inhibit cell-free virus particles, HFFs were seeded on 96-well microplates (Cellstar, Greiner Bio-One) at a density of $1.5 \times 10^4$ cells per well. The next day, aliquots of HCMV-TB40/E-BAC4 were preincubated with 1/50 diluted sera for 2 h at 37 °C, and the mixture was added to the HFF cultures for overnight incubation at 37 °C. Cells were then fixed with 80% acetone, and IE antigen was detected via immunofluorescence staining.

2.7. Analysis of Single-Particle Transmission in Cell-Associated Spread

HFFs were seeded on a 96-well microplate (Cellstar, Greiner Bio-One) at a density of $1.5 \times 10^4$ cells per well. The next day, cells were infected with Merlin-pAL1502-UL32EGFP-UL100mCherry at a very low infection multiplicity (MOI), resulting in around 0.2% infected cells per well. One day p.i., supernatants were removed and plasma added to the infected cells at a 50-fold dilution. After 5 d p.i., cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 30 min and permeabilized with a permeabilization solution (PBS with 10% sucrose (Sigma-Aldrich), 1% fetal bovine serum (PAN Biotech), and 0.5% Nonidet P40 (Sigma-Aldrich)) for 10 min, both at ambient temperature. The red fluorescent mCherry signal was enhanced by additional staining with a rabbit anti-dsRed polyclonal antibody (Clontech, Kusatsu, Japan), and Cy3-conjugated goat anti-rabbit IgG F(ab’)2 (Jackson ImmunoResearch). Nuclei were counterstained with DAPI (Sigma-Aldrich). Images were taken with a fluorescence microscope (Axioobserver D1; Zeiss) at a 640-fold magnification.

3. Results

3.1. Generation of Reporter Virus for High-Throughput Analysis of Cell-Associated Spread

3.1.1. Cloning of Reporter Virus Merlin-pAL1502-GLuc

For the generation of a reporter virus that would facilitate the screening of a blood-donor population for cell-to-cell spread-inhibiting antibodies, we used the genetic backbone of Merlin-BAC pAL1502 [28]. This Merlin derivative contained tet-operator sequences in front of the RL13 and UL128 genes, which are known to restrict the cell-free spread of HCMV. In HFFF-tet cells harboring the tet repressor, RL13 and UL128 are conditionally repressed, which allows release of cell-free infectivity into the supernatant and prevents the selection of mutants in these genes. In primary human cells lacking the tet repressor, RL13 and UL128 are expressed, and the virus grows in a strictly cell-associated manner. Using seamless mutagenesis, a GLuc expression cassette was inserted into the Merlin-pAL1502-BAC, downstream of the US34A gene. This insertion site was chosen to minimize the risk of interference with other open reading frames as it has been reported to have very low transcriptional activity [55]. Like the parental virus, this mutant grew in a cell-free mode in HFFF-tet cells after transfection of the recombinant BAC genomes. In contrast, when primary cells were infected with such cell-free virus stocks, the virus then grew in a strictly cell-associated focal manner.
3.1.2. Correlation between GLuc Activity and Focal Growth

To test whether GLuc activity correlates with the extent of focal growth, HFFs (2.0 × 10⁴ cells per well) were infected with the Merlin-pAL1502-GLuc in serial dilutions at MOIs of 0.00025–0.004 FFU/cell, and viral spreading was allowed for a total of six days. At 1 d p.i., either HCMV-seronegative human plasma was added at a 50-fold dilution for uninhibited growth, or direct-acting antivirals ganciclovir (800 μM) or BDCRB (0.04 mM) were added as inhibitors of focal growth. After four days (i.e., 5 d p.i.), supernatants were replaced with MEM5 and further incubated overnight. At 6 d p.i., GLuc activities were measured in the supernatants, whereas the cells were fixed, stained for IE antigens by immunofluorescence, and analyzed regarding the numbers of foci per well (Figure 1). As expected, increasing focus numbers in the IE-Ag staining correlated with increasing Gaussia luciferase signals in a linear relationship (Figure 1A), and inhibition of focal growth by the direct-acting antivirals was reliably detected by greatly decreased GLuc activity as compared to the uninhibited control (Figure 1B). Interestingly, nucleoside analog ganciclovir, which inhibits genome replication, repeatedly yielded lower GLuc activities than terminase inhibitor BDCRB, which targets packaging of viral genomes, indicating that the luciferase readout can stably report even minor differences. At the lowest concentration of the reporter virus, six foci per well were present, as determined by immunofluorescence staining, and this low focus number was still sufficient to provide a reliable luciferase signal. In conclusion, these experiments proved that the Merlin-pAL1502-GLuc assay is reliable and sensitive enough to screen potential inhibitors regarding their effect on cell-associated focal spread of HCMV.

Figure 1. Inhibition of focal growth reflected by Gaussia luciferase (GLuc) activity. Human foreskin fibroblasts (HFFs) were seeded and infected with Merlin-pAL1502-GLuc in a dilution series in the range of 0.00025–0.004 focus-forming units/cell (FFU/cell) resulting in six to 80 infected foci per well. The following day, infected cell cultures were treated with either human cytomegalovirus (HCMV)-seronegative human plasma, or two direct-acting antivirals ganciclovir (GCV) or 2-Bromo-5,6-dichloro-1-β-d-ribofuranosyl benzimidazole (BDCRB). Six days after infection, viral immediate-early (IE) antigens were detected by immunofluorescence staining to determine the number of infected foci per well. Additionally, the GLuc activity of cell-culture supernatants was measured. (A) Correlation between immunofluorescence-based readout and luciferase readout. (B) Detection of reduced focal growth in antiviral-treated cultures by the luciferase readout at various infection multiplicities. Bars represent the mean values of triplicates and error bars indicate the standard error of the mean (SEM).

3.2. No Cell-To-Cell Spread Inhibitors Identified in Large Cohort of HCMV-Seropositive Individuals

Plasma samples of 8400 HCMV-seropositive blood donors, which we had previously characterized regarding their neutralizing effect on cell-free viruses [37], were now screened with the GLuc-based
reporter assay concerning their capacities to inhibit cell-to-cell spread. Direct-acting antiviral agents and HCMV-seronegative plasma were included as positive and negative controls, respectively. Fibroblasts, seeded two days before, were infected with Merlin-pAL1502-GLuc at very low MOIs (0.002 FFU/cell). Plasma samples and control agents were added 1 d p.i., and incubated with the infected cells for four days. Cultures were then washed, further incubated overnight, and supernatants were harvested at 6 d p.i. for analysis of their relative luciferase activities. A total of 8034 samples passed the controls and could be evaluated.

Figure 2. Plasma from HCMV-seropositive donors does not inhibit cell-associated viral spread. HFFs were seeded on microplates and infected with Merlin-pAL1502-GLuc at infection multiplicities of 0.002 FFU/cell. The day after infection (p.i.), plasma samples were added at a 50-fold dilution, and cells were incubated further. On day 5 p.i., medium was exchanged, and on day 6 p.i., supernatants were harvested for analysis of luciferase activities. Two direct-acting antivirals (GCV and BDCRB) served as positive controls for inhibition of cell-associated HCMV spread. (A) GLuc activities of individual plasma samples divided by the mean value of whole plate level (i.e., relative luciferase activity). Each circle represents plasma from one of 8034 HCMV-seropositive donors. The median of relative GLuc activities of all tested samples is depicted by a dashed horizontal line and represents unrestricted focal growth. The lower dashed line reflects maximal reduction of focal growth by direct-acting antivirals. (B) Among the 14 plasma samples that showed the highest reduction of GLuc activity, seven samples were randomly selected for a retest via immunofluorescence detection of viral IE antigens, but none of them had an effect on focal growth as compared to plasma from an HCMV-seronegative donor. Bars represent mean values of 10 foci, error bars represent the SEM. (C) Representative images from three of these seven retested plasmas are shown.

The mean relative luciferase activity of the antiviral controls was around 0.12 (=12%), reflecting maximal inhibition of focal growth. None of the plasma samples reached this value, indicating that
none of them could substantially block cell-to-cell spread of the Merlin strain (Figure 2A). Nevertheless, seven out of the 14 top scorers in the luciferase screening were randomly selected (representing relative luciferase activities between 0.22 and 0.50) and re-evaluated for cell-to-cell spread inhibition in an immunofluorescence-based assay that visualized viral IE antigen in the nuclei of infected cells and thereby allowed to assess focus size at 6 d p.i.. None of these seven plasma samples notably reduced focal growth of strain Merlin when compared to HCMV-seronegative control plasma, whereas formation of foci was completely blocked by direct-acting antivirals (Figure 2B,C). Obviously, the few hits that had at least a partial effect in the screening assay represented statistical outliers rather than true biological activity against the cell-associated spread of HCMV.

3.3. Cell-Type-Dependent Differences in the Degree of Resistance against Neutralizing Sera

For our screening approach, we used plasma samples from the same donor population that we had previously tested regarding the neutralization of a cell-free virus [37]. It was remarkable that none of the “elite” donors with high and broad neutralization capacities against cell-free HCMV strains showed a notable effect on cell-to-cell spread of our reporter virus. To exclude the possibility of strain-specific resistance of the Merlin derivative that we used in the screening approach, we tested six of these highly neutralizing serum samples in a targeted approach against three strictly cell-associated recent HCMV isolates. To assess whether our fluorescence-based focus expansion assay was able to detect inhibition of focal growth in principle, we also included endothelial cells. Cell-associated HCMV spread in epi- and endothelial cells has repeatedly been reported to be at least partially sensitive to inhibitory antibodies with some virus strains [31,32,34,35]; hence, we could expect partial effects of our highly neutralizing serum samples in these cell types.

HFFs infected with the clinical isolates were cocultured for 6 d with an excess of HFFs or HEC-LTTs in the presence of sera known to have a high neutralizing capacity. Focal growth was visualized by indirect immunofluorescence staining of viral immediate-early antigens, and the number of antigen-positive nuclei per focus was evaluated as a readout for spreading efficiency of the virus under the various conditions. Focus formation was almost completely inhibited by the direct-acting antiviral agent ganciclovir, which served as a positive control. In the presence of serum from an HCMV-seronegative individual, which served as a negative control for unimpeded spread, large foci containing around 100 infected cells formed in HFF cocultures. Compared with this negative control, none of the six highly neutralizing sera could notably reduce the focus size in HFFs (Figure 3A,C), while their neutralizing capacity was concurrently confirmed by a complete inhibition of the cell-free virus (Figure 3B).

In HEC-LTT cocultures, the isolates formed smaller foci of around 20, 14, or four infected cells in the presence of HCMV-seronegative serum. As expected, some of the highly neutralizing sera partially inhibited the focus expansion in this cell type. Depending on the serum and the isolate, focus size was reduced to a variable degree, with about 50% reduction being the maximal effect that we observed (Figure 4A). These sera reduced not only the focus size but also the number of foci. However, as the foci were often not very confined, we considered that counting the total number of infected cells may be more accurate to evaluate the overall inhibitory effect. Therefore, we determined the total number of infected cells per well, showing that four out of six highly neutralizing sera significantly inhibited growth of clinical isolates in endothelial cells by 50% to 60% (Figure 4B; p values between 0.005 and 0.02), whereas one serum completely failed in this cell type, and reduction by one serum was not significant. To exclude the possibility that the inhibitory effect on focal growth in endothelial cells was due to the release of free virus in this cell type, we tested supernatants of endothelial-cell cocultures, but never found indications of cell-free infectivity with any of the isolates.
Figure 3. Sera with highly neutralizing capacity against cell-free HCMV transmission cannot inhibit cell-associated spread of clinical isolates in fibroblasts. (A) Fibroblasts infected with cell-associated clinical HCMV isolates were cocultured with an excess of uninfected HFFs, and, the following day, sera with highly neutralizing capacity against cell-free HCMV transmission were added to the coculture. Addition of seronegative human serum or a direct-acting antiviral served as a negative or positive control, respectively. Cocultures were then incubated for six days more to allow for cell-associated viral spread, followed by fluorescence staining for viral IE antigens (red signal). Nuclei of cells were stained with DAPI (blue signals). Three representatives out of six tested sera are shown. (B) To prove their neutralizing capacity, sera were mixed with HCMV-TB40/E-BAC4 (MOI 1) and incubated for 2 h. Fibroblasts were then infected with the serum-virus mixture. The next day, IE antigens were detected via indirect immunofluorescence staining (red signals). (C) For the three sera shown in panel A, focus areas were determined and compared with the negative control. For each serum, the bar represents the mean value of the three isolates (four to seven foci evaluated per isolate). Error bars represent the SEM.

In summary, even exceptionally effective neutralizing sera could not inhibit the cell-associated spread of HCMV in fibroblasts, while the majority had the expected partial effect on cell-associated spread in endothelial cells.

3.4. Neutralizing Serum Fails to Reduce Cell-To-Cell Transmission of HCMV Particles

A possible explanation for viral cell-to-cell spread, despite the presence of highly neutralizing anti-HCMV antibodies, might be provided by the assumption that numerous virions are transmitted during cell-associated spread, resembling infection at high MOIs [56]. Based on this consideration, we hypothesized that highly potent neutralizing antibodies may reduce the number of transferred
particles, but may not be sufficient to prevent infection, which can be accomplished by a single virion that is not inhibited.

**Figure 4.** Highly neutralizing sera can partially reduce cell-associated spread of HCMV in endothelial cells. (A) Fibroblasts infected with cell-associated clinical HCMV isolates were cocultured with an excess of endothelial cells (HEC-LTTs) and, the following day, sera from highly neutralizing blood donors were added to the coculture. Addition of seronegative human serum and a direct-acting antiviral served as a negative and positive control, respectively. The cocultures were then incubated for six days more to allow for cell-associated viral spread, followed by fluorescence staining for viral IE antigens (red signal). Nuclei of cells were stained with DAPI (blue signals). Three representatives out of six tested sera are shown. (B) To quantify the effect of sera on focal growth, the overall numbers of infected cells per well were counted and divided by the number of infected cells cultured in HCMV-seronegative serum (neg). Each bar represents the mean value obtained with three different clinical isolates. Error bars reflect the SEM. Asterisks indicate significant (*) or highly significant (**) differences as compared with the control (neg).
To enable investigation of cell-associated HCMV spread on a single-particle level, a dual-labeled HCMV was generated on the genetic background of HCMV clinical isolate-like strain Merlin. The envelope glycoprotein M (gM) was labeled by insertion of the red fluorescent protein mCherry directly after the first transmembrane region, and EGFP was fused to the C-terminus of the capsid-bound tegument protein pp150. When coculture experiments are performed with this dual-fluorescent mutant, virus particles that are transferred from a late-stage infected cell to neighboring cells can be detected and enveloped particles (red and green signal, yellow dot-like speckles) can be distinguished from naked viral capsids (green dot-like speckles). This virus was then employed to analyze the effect of neutralizing plasmas on the number of virions transferred during cell-to-cell spread. HFFs were infected with the dual-labeled Merlin, at 1 d p.i. plasma was added, and, at 5 d p.i., cells were fixed, and the red fluorescent signal was enhanced via indirect immunofluorescence for easier evaluation. Using this approach, we tested nine plasma samples with previously identified potent neutralizing capacity [37] and compared them with plasma from an HCMV-seronegative individual as a negative control.

First of all, our findings with the seronegative plasma supported the assumption of high infection multiplicity during cell-to-cell transmission of HCMV. Between 10 and more than 50 virions were found on cells in the surroundings of late-stage infected cells, and the majority of these virus particles accumulated at the nuclei of the respective cells. Most of them did not contain an envelope signal, as indicated by the fact that they only had a green signal but lacked red fluorescence (Figure 5).

**Figure 5.** Highly neutralizing plasma does not reduce the number of transferred viral particles. Fibroblasts were infected with the dual-labeled Merlin-pAL1502-UL100mCherry-UL32EGFP, and treated either with plasma with highly neutralizing capacity against cell-free HCMV transmission or with HCMV-seronegative plasma on the following day. Five days after infection, cells were fixed, mCherry signals were enhanced with indirect immunofluorescence, and cultures were investigated with a fluorescence microscope. A representative image of each condition is shown. The viral capsid-associated tegument protein pUL32 is visualized in the green channel, whereas viral envelope protein gM is visualized in the red channel. Dot-like dual-fluorescent signals appear yellow in the merged image and represent enveloped viral particles, whereas naked viral capsids are represented by green-only dot-like signals. Nuclei were counterstained with DAPI (grey).
Few virus particles showed red and green signals, suggesting that complete virion particles were released by the productively infected central cell, and that such particles lost their envelope during penetration into the neighboring cell. This interpretation was further corroborated by the accumulation of red fluorescent gM signals at a perinuclear site in cells where many green particles were detected at the nucleus. Regarding a possible effect of neutralizing plasmas, we could not observe differences in the number of transferred viral particles when the nine highly neutralizing plasma samples were compared with the negative control. This led us to reject our hypothesis that the resistance of cell-to-cell spread against neutralizing plasmas is due to a greatly reduced but still-sufficient particle transfer in the presence of such plasma. Obviously, cell-associated virus transfer is completely unaffected by anti-HCMV antibodies contained in such plasma samples.

4. Discussion

Directly after isolation from clinical specimens, HCMV grows focally in fibroblast cultures without detectable cell-free infectivity in the supernatant [26,27]. It is, therefore, tempting to speculate that in vivo HCMV also can spread in a cell-associated fashion, something that is supported by the lack of detection of cell-free virions in plasma, as well as the ability of leucodepletion to prevent virus spread [39–41]. During adaptation to cell culture, viral gene loci RL13 and UL128L are modified, infectious progeny becomes detectable in the supernatant, and the virus can now spread both in a cell-associated and a cell-free fashion [28,29,36]. Cell-free and cell-associated spread are usually distinguished by the inhibitory effect of neutralizing antibodies: the antibody-sensitive part of the infectivity is regarded “cell-free”, whereas the antibody-resistant part is regarded as “cell-associated” [57]. Fitting with this definition, recent HCMV isolates that do not release detectable infectivity into the supernatant (as well as isolates engineered to retain the cell-associated phenotype of clinical isolates) are almost completely resistant to neutralizing antibodies [30,36,58], suggesting a fundamental mechanistic difference between the two transmission modes.

The disappointing results with the clinical application of antibodies for the treatment of HCMV infection may at least in part be explained by the assumption that HCMV can spread in a cell-associated fashion in vivo, and that this mode of transmission cannot be prevented by anti-HCMV immunoglobulin preparations. In line with that, the partial protection elicited by a recombinant gB vaccine is obviously not mediated by increased capacity of antibodies to inhibit cell-associated spread [23]. This is, to some extent, surprising because the core fusion machinery of HCMV, which is targeted by neutralizing antibodies, is not only required for cell-free but also for cell-associated spread. This can be concluded from the fact that mutants lacking gB, gH, and gL cannot be reconstituted at all, whereas mutants lacking gO can form small plaques because only cell-free infectivity is specifically affected [59–61]. It is, therefore, rational to propose that, in principle, viral fusion machinery can also be targeted by inhibitory antibodies to prevent cell-to-cell spread. Resistance of recent clinical isolates against currently available anti-HCMV immunoglobulins, however, indicates that these preparations have either too little potency or the wrong specificity to be effective against cell-associated focal spread.

Previous work showed that activity against cell-associated spread is not common in sera from HCMV-seropositive individuals or pooled preparations like hyperimmunoglobulins [30,36,58], which did, however, not exclude the possibility that a few individuals might have exceptionally potent antibodies in their plasma that can inhibit the cell-associated spread of HCMV. To test this possibility, we aimed to apply a high throughput approach for detection of cell-to-cell inhibitory antibodies to a large blood-donor population that we had recently analyzed for neutralizing capacities against a cell-free virus [37]. Therefore, a Gaussia luciferase-secreting reporter virus was generated on the genetic background of a variant of the Merlin strain that contains intact RL13 and UL128L regions and, therefore, spreads in a cell-associated fashion like recent clinical isolates [28]. Luciferase activity in the supernatant of infected cultures was shown to reflect cell-associated focal growth of the virus, and hence was a valid readout to screen for donors that can reduce this mode of transmission by their plasma.
Remarkably, none of the 8400 plasma samples that we screened could block the focal spread of the Merlin reporter virus to a similar extent as the direct-acting antiviral agents that we used as controls. Furthermore, when the samples with the lowest luciferase values in the screening were retested and additionally evaluated in an independent, more accurate fluorescence-based assay, not even a partial effect on viral spread could be reproduced, suggesting that their low values in the initial screening were due to test variation rather than a real antiviral effect. This surprisingly clear negative finding emphasizes how effectively HCMV escapes from antiviral antibody responses by its cell-associated transmission mode. To exclude the possibility that this is a strain-specific effect, we tested the inhibitory effect of six sera with high neutralizing capacity against cell-free HCMV on three recent cell-associated patient isolates and showed that none of the six sera could inhibit cell-associated spread of these isolates in fibroblasts at concentrations that completely blocked cell-free transmission of strain TB40/E. Concerning our initial working hypothesis, this means that, if donors with cell-to-cell-inhibiting plasma exist at all, they are extremely rare, a result that is consistent with the inability of pooled antibodies from HCMV-seropositive donors to inhibit cell-associated transfer into multiple cell types [36].

This clear failure of anti-HCMV sera regarding inhibition of a cell-associated virus spread is hard to reconcile with the notion that cell-to-cell spread depends on the same glycoproteins that are necessary for cell-free infection. One possible explanation could be that cell-associated spread occurs at high infection multiplicity [56]. If numerous virus particles were transmitted from productively infected cells to their neighboring cells in a growing focus, and antibodies neutralized the vast majority of such particles, a single successful particle would still be sufficient to initiate the replication cycle in the neighboring cell. In this scenario, the reason for antibody resistance would be a quantitative rather than a qualitative issue. To address this, we generated a dual-fluorescent mutant of the cell-associated Merlin strain, in which viral envelope protein gM was tagged with red fluorescent protein mCherry and the capsid-associated tegument protein pp150 was tagged with green fluorescent protein EGFP [62]. As expected, infection multiplicity in a growing focus was high, with several dozens of virions being transferred from the productively infected cells to their neighboring cells. However, the presence of highly neutralizing antibodies did not reduce the number of virus particles. Given that the HCMV fusion machinery is necessary for cell-to-cell transmission of viral progeny, the simplest explanation is that the sites where neighboring cells interact during transmission are not accessible to antibodies, leaving the possibility that smaller fusion inhibitors might be more successful, e.g., small peptides derived from PDGFR-alpha [63] or small compound drugs that can interfere with the function of viral glycoproteins. Our test system can now also be applied to screen such compounds for their cell-to-cell spread-inhibiting activity.

To some extent, antibodies with highly neutralizing capacity against cell-free HCMV infection could inhibit cell-associated viral spread in endothelial cells, whereas they completely failed in fibroblasts. This is in agreement with previous reports that pentamer-dependent cell-to-cell spread (in epi- and endothelial cells) is more sensitive to anti-HCMV antibodies [31,32,34,35]. Inhibition was, however, never complete, even with the most potent neutralizing anti-HCMV sera. Assuming that partial resistance of pentamer-dependent spread and complete insensitivity of pentamer-independent spread also applies to the situation in vivo, strategies for inhibition of the cell-associated spread of HCMV are desired, as they might provide a basis to improve the clinical efficacy of anti-HCMV treatment regimens. Of course, direct-acting antivirals serve this purpose. However, in situations where their use is limited by adverse effects or the occurrence of resistant strains [11,12], alternatives with a different mode of action may be required.

It is important to mention that our results do not absolutely exclude the possibility that antibodies might be effective against cell-associated spread in fibroblasts, but they indicate that such antibodies cannot be detected easily in donor plasma or serum. Still, a possibility remains that certain antibody specificities exist that are underrepresented in whole serum and, therefore, not concentrated enough to exert their effect in our screening assay. To search for such specificities, cloning of complementarity-determining regions from individual B cells [64], followed by screening
with the Gaussia luciferase reporter virus, might be an option. These results also do not argue against a contribution of the humoral immune response in general for the control of HCMV. It is conceivable that neutralizing antibodies can be beneficial at certain steps of the pathogenesis, e.g., the initial transfer of the virus from host to host, and that more indirect effects of anti-HCMV antibodies may help control viral spread in interplay with other components of the immune system, as in antibody-dependent cellular cytotoxicity (ADCC).

5. Conclusions

Screening of a large blood-donor population with a Merlin-derived reporter virus could not identify individuals that can inhibit the cell-associated spread of HCMV by their plasma, underscoring the remarkable capability of HCMV to evade the host’s antibody response via this transmission mode. Future attempts to inhibit cell-to-cell transmission of HCMV particles may, therefore, need to focus on smaller molecules to facilitate access or on exceptional antibody specificities, which are usually underrepresented in donor plasma.

Successful treatment of HCMV spread should target both cell-free as well as cell-associated spread. In previous work, we demonstrated that a small subpopulation of blood donors carry antibodies with high neutralizing capacity in vitro that might also reduce cell-free spread in vivo. Since this study shows the limitations of an antibody-based approach regarding cell-associated spread, future studies should explore combinations of antibodies with high neutralizing capacity and other pharmacological interventions targeting focal spread.


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