Phenotypic characterization of human cytomegalovirus strains in cell cultures based on their transmission kinetics

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We established a new ‘transmission kinetic assay (TKA)’ to quantify the human cytomegalovirus (HCMV) transmission between cells in vitro and to phenotypically characterize HCMV strains based on their mode of transmission by flow cytometric analysis. On one hand we used the genetically modified HCMV strain TB40/E-delUL16-GFP, and on the other hand, clinical isolates. When twofold diluted infecting cells were seeded to a constant number of uninfected cells, the transmission of virus on each day (day 0–5) followed a strictly linear pattern, which was characterized by a linear equation. The slope of this linear equation represents ‘the number of newly infected cells per infecting cell’. To standardize the TKA, the slopes of the different days were plotted against the corresponding days. This resulted in a new linear equation with a new slope value, which characterizes the transmission kinetics. To differentiate cell-associated and cell-free modes of transmission, we introduced HCMV neutralizing antibodies into the system. The slope was 0.9 (±0.5) when the virus exhibited only cell-associated transmission and was 4.1 (±0.7) when the virus exhibited both modes of transmission. TKA was then applied to different clinical isolates and they were phenotypically characterized based on their modes of transmission. Apart from the quantitative analysis of HCMV transmission and the phenotypic characterization of clinical isolates, the TKA was applied to quantify the inhibition of clinical isolates transmission by immune cells and to study the effect of cytokine (IL-2) on immune cells inhibiting HCMV transmission.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the Betaherpesvirinae subfamily, is a widespread pathogen. Seroprevalence in adults ranges from about 45 to 100 %, increasing with age and varying with socio-economic background (Cannon et al., 2010; Griffiths, 2012). HCMV is the predominant infectious reason for health abnormalities in congenitally infected children (Cheeran et al., 2009). Infection with HCMV is mostly asymptomatic in immunocompetent individuals, but the virus is never cleared and establishes latent infection for the lifetime of its host with possible reactivations (Sinclair et al., 2006; Wills et al., 2015). Primary infection or reactivation in immunocompromised individuals, such as transplant recipients, results in considerable morbidity and mortality (Emery, 1999; Gerna et al., 1996).

Following initial HCMV infection, transmission of the virus to neighbouring cells can occur by the virus released from infected cells (cell-free mode) or by direct cell-to-cell contact of the infected cell with neighbouring cells (cell-associated mode) (Sattentau, 2008; Wu et al., 2015). Transmission of primary clinical HCMV isolates in cell cultures is presumed to be highly cell-associated (Digel et al., 2006; Sinzger et al., 1999; Yamane et al., 1983) and it is generally assumed that this way of transmission is of great importance in vivo. Highly passaged laboratory HCMV strains are transmitted by both, cell-free and cell-associated mode. This was shown to depend upon mutations in RL13 and UL128 locus (Dargan et al., 2010; Stanton et al., 2010; Prichard et al., 2001; Adler et al., 2006).

Due to the strict species specificity of this virus (Osborn 1982; Mocarski 1996), animal models of HCMV infections are not available to study pathogenesis or to assess antiviral drugs. Thus, by necessity, most studies were performed in in vitro systems. Although differentiated genotypic characterization of HCMV is routinely possible today, a spectrum of exact quantitative biological methods is still needed for a dynamic phenotypic characterization of virus strains. Even though there are some titration methods to examine the viral replication and virus infection in cells (Kahl et al., 2000; Sinzger et al., 1997), practical methods do not exist to exactly quantify the HCMV transmission in cell cultures.

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One supplementary figure is available with the online Supplementary Material.
Fig. 1. Kinetics of HCMV transmission in fibroblast and epithelial cells. (a) Live images of transmission using a recombinant HCMV expressing GFP (TB40/E-delUL16-GFP) in fibroblast (HFF) and epithelial (ARPE-19) cells, without and with HCMV neutralizing antibodies (HCMV Ab) on different days. (b and d) Graphs showing percentage of infected cells on different days. (c and e) Transmission kinetic assay.
HCMV transmission can be studied in fibroblasts and epithelial cells. We named the new assay ‘transmission kinetic assay (TKA)’. During HCMV infection, the virus encounters the host’s innate and adaptive immunity. The clinical outcome of HCMV infection mainly depends on the antiviral immunity. Although there are some methods available to determine the inhibition efficiency on HCMV infection by immune cells, such as plaque reduction assay and cytotoxic T lymphocyte (CTL) assay (Landry et al., 2000; Tabi et al., 2001; Sinzger et al., 2007), assays to quantify the inhibition are still needed. The newly established TKA protocol allows the comparative quantitative analysis of the inhibition of HCMV transmission by effector cells of both innate and adaptive immunity.

In this study, we used the TKA to firstly quantify the HCMV transmission to phenotypically characterize HCMV strains based on their modes of transmission. We then applied the same TKA to quantify the inhibition of HCMV transmission provided by different immune cells and the effect of cytokine on these immune cells.

RESULTS

Quantification of human cytomegalovirus transmission in fibroblasts and epithelial cells

HCMV transmission can be studied in vitro by adding a limited number of infecting cells to a large number of uninfected cells. The seeded infecting cells can transmit virus to the surrounding cells either by virus released into the supernatant (cell-free mode) or by cell-to-cell contact (cell-associated mode). In the case of the virus spreading only by cell-associated transmission and thus leading to a focus surrounding the infecting cell, its kinetics can be determined by counting the infected cells per focus at different time points after infection (Wu et al., 2015; Sinzger et al., 1997, 2007). However, this manual counting method is extremely labour intensive and cannot be applied to quantify the transmission when cell-free virus transmission is predominant. Therefore, we established a new quantitative assay to determine the HCMV transmission based on counting by FACS.

In order to easily track live HCMV transmission and to set up the TKA, we used a UL16 deletion mutant in the background of TB40/E expressing GFP (TB40/E-delUL16-GFP). This particular UL16 deletion mutant was selected due to its bright GFP expression compared to all other mutants tagged with GFP available to us. GFP which is under the control of the UL16 (early) gene promoter was detectable 5 h post-infection (hpi). The detection of GFP in cells is used as a marker for early gene expression of HCMV, which represents infection. We added TB40/E-delUL16-GFP-infected human foreskin fibroblasts (HFF) to uninfected HFF or uninfected retinal pigment arterial epithelial cells (ARPE-19) and took live images of the cells on days 0, 3 and 5 (Fig. 1a). In order to verify whether our TKA can be used to quantify the transmission in different cell types, we used epithelial cells and fibroblasts. As shown in the first row of Fig. 1(a), the GFP signals indicate that the number of infected cells in both cell types, increased with time after infection.

It was shown that the cell-free virus transmission can be blocked by neutralizing HCMV antibodies (HCMV Ab) and they have no effect on cell-associated transmission in fibroblasts (Gerna et al., 2008; Jacob et al., 2013). Hence, we used the HCMV Ab (IgG, commercially available ‘Gamunex’) as a tool to distinguish the cell-free and the cell-associated modes of transmission in this UL16 deletion mutant. Immunofluorescence images of fibroblast cultures showed a reduction in the GFP-positive cells in the presence of HCMV antibodies (Fig. 1a, left panel, second row). This reduction presumably represented the fraction of cell-free transmission. In order to verify this, the supernatants of the cultures from day 5, with and without HCMV Ab, were titrated on fibroblasts to check for cell-free infectivity. Cell-free infectivity was detected only in the absence of HCMV antibodies (day 5, 3×10^3 p.f.u. ml^-1, mean value of three experiments). In contrast, the transmission from infecting fibroblasts to epithelial cells was completely blocked in the presence of neutralizing antibodies (Fig. 1a, right). Thus, we concluded that cell-associated transmission did not occur in epithelial cells when infected fibroblasts were used for infection in the presence of neutralizing antibodies. In our assay, this condition was taken as a positive control for the effect of antibodies.

The increase of HCMV infected cells over time or the reduction of HCMV transmission in the presence of antibodies can be observed by life cell imaging (fluorescence microscopy). However, as said earlier, the quantification of the transmission becomes difficult, especially when the virus exhibits the cell-free mode of transmission. According to our experiments, the more infecting cells were applied, the lower the ratio became between the infecting and the...
infected cells. This may be explained by different numbers of uninfected cells surrounding the infecting cells or different cell cycle stages of the neighbouring uninfected cells. Thus, it is reasonable to assume that the number of infecting cells in relation to the uninfected cells is relevant for the determination of the kinetics of virus transmission. In order to verify this, we inoculated different numbers of infecting cells after twofold (640, 320, 160, 80, 40, 20 and 10) or fourfold dilutions (640, 160 and 40), with always the same number of uninfected cells (20000). The infected cells were counted by FACS on every day (day 0–6). Since the FACS results were in percentages (Fig. 1b, y-axis), we presented the 'number' of infecting cells seeded on day 0 as 'percentage' of total cells (3.2, 1.6, 0.8, 0.4, 0.2, 0.1 and 0.05 %) to ease data presentation and interpretation (Fig. 1b, x-axis). The number of seeded infecting cells as percentage of total cells is calculated as follows:

\[
\left( \frac{\text{Number of seeded infecting cells}}{\text{Total number of uninfected cells}} \right) \times 100 = \left( \frac{640}{20000} \right) \times 100 = 3.2\%
\]

In Fig. 1(b, d), the percentages of infected cells on different days (y-axis) obtained from FACS were plotted against the percentages of infecting cells added on day 0 (x-axis). These experiments were performed with and without the HCMV Ab. As shown in Fig. 1(b), the percentage of infected cells on each day correlated linearly with the percentage of infecting cells seeded on day 0 in both conditions. One exemption was found on day 6 in the absence of HCMV Ab. Here the curve was sigmoidal due to the lack of uninfected cells (Fig. 1b, left). Therefore, we evaluated the transmission only from day 0 to 5.

Linear equations (\(y=mx+C\)) were generated for each linear line (GraphPad Prism 5). The slope of each line is represented by 'm' in the equation and 'C' represents the intercept. Generally, the intercept values are considered as the error values of the linear line. Therefore, only the slope values were considered for determining the transmission kinetics. Using the obtained slope values, i.e. from the linear correlation between x- and y-axis, the assay became independent of the individual numbers of infecting cells as long as the same twofold dilution series was maintained. This means that irrespective of titration of infecting cells from 500 or 1000 cells, the slope remains the same, provided that the twofold (fourfold and eightfold) dilution series is maintained. From the classical slope equation (change in y-axis to change in x-axis), the slope in our assay represents the 'number of newly infected cells per infecting cell' on a given day.

\[
\text{Slope} = \frac{\Delta y}{\Delta x} = \frac{\text{change in % of newly infected cells per infecting cell}}{\text{change in % of seeded infecting cells on day 0}}
\]

e.g. slopes for day 3 shown for any two points (Fig. 1b, blue lines)

\[
\frac{(26 - 15)\%}{(2 - 1)\%} = \frac{11\%}{1\%}
\]

The dimension of both values is percent (of total cells). The final value becomes an absolute value when substituting percent by the number of total cells (e.g. 20 000).

\[
\frac{(11 \times 20000)}{100} = \frac{11}{T}
\]

This means 11 uninfected cells are newly infected cells on day 3 from every infecting cell seeded on day 0. Therefore, the slope represents the 'number of newly infected cells per infecting cell'.

By comparing the slopes of the TKA carried out with and without HCMV Ab, we can clearly show that the kinetics of HCMV transmission was retarded if only cell-associated transmission was allowed (Fig. 1b, right) and faster if the virus used both, cell-free and cell-associated transmission (Fig. 1b, left). Moreover, the HCMV transmission was much slower in epithelial cells than in fibroblasts (Fig. 1b, left and Fig. 1d, left). Furthermore, in epithelial cells HCMV transmission was totally inhibited by HCMV Ab (Fig. 1d, right).

To obtain the typical kinetic graph showing changes over time, we plotted 'the number of newly infected cells per infecting cell' of each day [slopes from Fig. 1(b, d)] against the corresponding days. This resulted in Fig. 1(c) (from three different experiments) and Fig. 1(e) (from 2 different experiments), where the differences in transmission kinetics induced by the addition of HCMV Ab can be clearly seen for HFF (Fig. 1c) and ARPE-19 cells (Fig. 1e). Since the kinetics again followed a linear regression, we took the slope generated for these linear graphs as the final transmission kinetic value. The slope value was high (around 4) when both cell-free and cell-associated transmission (in the absence of HCMV Ab) were involved. The slope value was low (around 1), when only cell-associated transmission (in the presence of HCMV Ab) was involved (Fig. 1c). The detailed TKA protocol is shown in the flowchart in Fig. S1 (available in the online Supplementary Material).

With the TKA, HCMV transmission was quantified and also the different modes of transmission in cell cultures could be discriminated using the obtained transmission kinetic value.

**Quantification of human cytomegalovirus transmission kinetics using different markers**

Although live cell analysis was possible using the GFP expression, this method cannot be used to track the transmission in HCMV strains, unless they are genetically modified.
Therefore, HCMV antigens expressed in infected cells were required for quantification. Detection of immediate early antigens (IEAs) is most widely used to trace HCMV infection. IEA is expressed earlier (2 hpi) than the GFP signal under the control of UL16 promoter (5 hpi). We compared IEA expression and GFP detection in fibroblasts by indirect fluorescence microscopy and FACS. Fig. 2(a) shows the comparison of IEA expression by indirect immunofluorescence and GFP expression of the same cells on days 0, 3 and 5 after infection. In addition, the inhibition of HCMV transmission by HCMV Ab can be similarly determined by using either the IEA or GFP as a marker (Fig. 2a, right). It was also possible to quantify the infected cells using IEA expression by FACS, since IEA were distributed homogenously throughout the nucleus of infected cells (Djaoud et al., 2013; Li et al., 2015). For FACS analysis, the cells were fixed, permeabilized (4 % PFA and 80 % ethanol) and stained with IEA antibodies in order to detect IEA and GFP simultaneously in separate channels of the flow cytometer. To determine

Fig. 2. Kinetics of transmission determined by IEA and GFP detection. (a) HCMV transmission in cells shown by live images (GFP) and IEA staining after acetone fixation. Transmission of infection to neighbouring cells was determined in the presence and the absence of HCMV Ab from day 0 to 5. (b) The number of newly infected cells per infecting cell determined by GFP and IEA detection with and without the addition of HCMV Ab conditions were plotted against the corresponding days after infection. The primary graphs as shown in Fig. 1(b, d) for IEA and GFP are not shown here. Error bars depict mean values±SD for two independent experiments. The slopes from the linear equations shown below the graph characterize the transmission kinetic value.
the transmission kinetics using IEA or GFP (Fig. 2b), the identical TKA protocol was used as described for Fig. 1 (b, d). By using IEA to define infected cells, HCMV transmission was marginally increased compared to the detection of the later expressed GFP. We also showed (Fig. 2b) that the transmission kinetic values (slopes) were similar when IEA and GFP were used as markers.

These results demonstrated that similar to GFP, IEA staining can also be used to determine the HCMV transmission kinetics. Thus, we showed the quantification of the HCMV transmission by two different markers.

Application of transmission kinetic assay for phenotypical characterization of clinical isolates

We applied the TKA to quantify the transmission kinetics of four clinical isolates which had previously shown different growth characteristics (Wu et al., 2015).

The clinical isolates were phenotypically characterized by using IEA as a marker to quantify HCMV transmission by FACS. Clinical isolates E30546 and E52812 clearly showed slower transmission kinetics with a slope around 1 in fibroblasts compared to clinical isolates E68240 and E56647 which exhibited a slope around 4 (Fig. 3). These slope values (around 1 and 4) were very similar to those obtained for the TB40/E-delUL16-GFP strain with and without the addition of HCMV Ab. This indicates that the clinical isolates E30546 and E52812 followed only the cell-associated mode and the clinical isolates E68240 and E56647 exhibited both modes.

In order to ascertain the mode of transmission, the clinical isolates E56647 and E30546 were treated with HCMV Ab and the cell-free virus was quantified in their supernatants. With the addition of antibodies, the transmission was highly reduced (62%) for the clinical isolate E56647 (Table 1b). In contrast, there was no detectable reduction in the transmission of clinical isolate E30546 in the presence of antibodies (Table 1b). When cell-free virus from the cultures of both clinical isolates was titrated, cell-free infectivity was found only in the supernatants of clinical isolate E56647 (day 5, 1×10^3 p.f.u. ml⁻¹). We concluded that the clinical isolate E30546 infected new target cells only by cell-associated transmission, whereas the clinical isolate E56647 infected cells by both, cell-free and cell-associated transmission.

To study the inter-test variability of our TKA, we evaluated the results of all experiments (Figs 1c, 2b and 3). The mean value of the transmission kinetics of the viruses exhibiting both, cell-free and cell-associated transmission modes was 4.1 (±0.7) but 0.9 (±0.5) for only cell-associated transmission. This means that a value of ~4 characterizes clinical isolates or virus strains that are transmitted by the cell-associated and cell-free modes and a value of ~1 applies to clinical isolates or strains that are transmitted only by the cell-associated mode. In conclusion, we established a robust assay to determine the mode of transmission of clinical HCMV isolates by quantification of their transmission kinetics.

Fig. 3. Kinetics of transmission of four HCMV clinical isolates in fibroblasts. The kinetics of transmission of four clinical isolates (E56647, E68240, E52812 and E30546) are represented by the slope of the linear equations below the graph which was obtained by plotting the number of newly infected cells per infecting cell (obtained by FACS quantification of IEA staining) against the corresponding days (same presentation as in Fig. 1c, e). The primary graphs as shown in Fig. 1b, d are not shown here. Error bars depict mean values±SD for two independent experiments.

Clinical isolates – transmission kinetics

<table>
<thead>
<tr>
<th>Clinical isolates</th>
<th>Linear equation</th>
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<tr>
<td>E56647</td>
<td>( y = 4.7455x + 1.1223 )</td>
</tr>
<tr>
<td>E68240</td>
<td>( y = 4.3274x + 0.8501 )</td>
</tr>
<tr>
<td>E30546</td>
<td>( y = 0.8085x + 0.8197 )</td>
</tr>
<tr>
<td>E52812</td>
<td>( y = 0.6841x + 1.1062 )</td>
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http://jgv.microbiologyresearch.org
Application of the transmission kinetic assay to quantify the inhibition of human cytomegalovirus transmission by cytokine-activated and non-activated immune effector cells

In order to verify whether our TKA can be applied to quantify the inhibition of transmission by immune effector cells, we used the same TB40/E-UL16-GFP strain in the presence of innate (NK cells) and adaptive (T cells) effector cells. We also applied IL-2-activated and non-activated immune cells from seropositive donors to analyse the effect of IL-2 on immune cells in inhibiting HCMV transmission.

The procedure of TKA was adapted with the following interventions: (i) NK cells (with and without IL-2 addition), (ii) pan-T cells (with and without IL-2 addition) and (iii) HCMV Ab as positive control. NK cells and pan-T cells were purified from peripheral blood mononuclear cells (PBMC) of two HCMV seropositive donors (E: T=0.25). As shown in Fig. 4, the transmission kinetic value was again around 4 for the condition without interventions similar to Fig. 1(c) and again, addition of HCMV antibodies restricted the transmission to a transmission kinetic value around 1. NK and T cells both controlled the HCMV transmission when stimulated with IL-2. NK cells show the highest control of HCMV transmission when stimulated with IL-2 (transmission kinetic value even lower than with HCMV antibodies), compared to T cells. Without IL-2 stimulation, there was only marginal control of HCMV transmission by NK cells. We also analysed the interferon-gamma (IFN-γ) secretion by T and NK cells in the supernatant on day 3 by ELISA. NK and T cells secreted around 400 and 210 pg ml⁻¹ IFN-γ, respectively, only when stimulated with IL-2. The concentration of IFN-γ was negative without IL-2 stimulation. This indicates that the difference in the reduction in HCMV transmission by NK and T cells stimulated by IL-2 could be due to IFN-γ. The effect seen for T cells might be partly due to the allogeneric system indicating a non CMV-specificity. The higher effect on the inhibition of transmission by NK cells in this assay is plausible since they are not dependent on MHC presented peptides.

We also applied our TKA method to verify the effect of immune cells in clinical isolates transmission exhibiting different modes of transmission. We used the two clinical isolates, E30546 which showed only the cell-associated mode of transmission and E56647 which exhibited both, the cell-associated and the cell-free mode of transmission.

NK and T cells were added to the TKA to study their effect on the different modes of transmission. Since the effects of NK and T cells were quite prominent in the presence of IL-2, we purified NK and pan-T cells from three seropositive donors and added them in the TKA only in the presence of IL-2 (E: T 0.25). We determined the inhibition of the transmission of the clinical isolates by immune cells on day 3. Only on day 3, a clear difference in the mode of transmission for both clinical isolates (Fig. 3) was detected and the effect of immune cells on HCMV transmission (TB40/E-delUL16-GFP) was higher compared to day 5 (Fig. 4). Linear equations obtained for different conditions on day 3 for both clinical isolates with different modes of transmission are shown in Table 1(a). The percentages of respective reductions on day 3 were calculated from the slopes (number of newly infected cells per infecting cell) under the following conditions, with (HCMV Ab, NK, T cells) and without (control) interventions (Table 1b).

The percentages were calculated according to the formula below.

For example, the inhibition of the clinical isolate E56647 by NK cells is:

\[
\text{Percentage inhibition} = \left( \frac{\text{Slope of control} - \text{Slope of NK}}{\text{Slope of control}} \right) \times 100
\]

NK cells showed a higher control of the transmission in

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<th>Table 1. Inhibition of clinical isolates transmission by immune cells</th>
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<td>(a) Linear equations obtained on day 3 for different interventions on two different clinical isolates</td>
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<tr>
<td>Clinical isolate E56647</td>
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<tr>
<td>Control (n=3)</td>
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<tr>
<td>HCMV antibody (n=3)</td>
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<td>T cells (n=3)</td>
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<td>NK cells (n=3)</td>
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<th>(b) Inhibition of clinical isolate transmission by interventions.</th>
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<td>HCMV antibody (%)</td>
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<td>T cells (%)</td>
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<td>NK cells (%)</td>
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both clinical isolates as compared to T cells. However, the inhibitions of the clinical isolate E56647 (mixed transmission phenotype) by NK cells (89%) and T cells (56%) (Table 1b, left) was also again higher as compared to the inhibition of the clinical isolate E30546 (cell-associated transmission mode) by NK cells (45%) and T cells (12%) (Table 1b, right).

In summary, the scope of TKA application covered:

1. Quantification of the inhibition provided by interventions like antibodies and immune cells on the HCMV transmission in cell cultures on different days.
2. Analysis of the effect of cytokines on immune cells in inhibiting HCMV transmission.
3. Quantification of the inhibition by immune cells on clinical isolates transmitted by different modes.

DISCUSSION

HCMV research cannot benefit from animal models due to its species specificity (Osborn, 1982; Mocarski, 1996). Hence, a spectrum of different methods is always needed to phenotypically characterize different HCMV strains in cell cultures. So far, different HCMV isolates or genotypes could only be phenotypically characterized based on few phenotypes (Kahl et al., 2000; Sinzger et al., 2007; Landry et al., 2000, Chevillotte et al., 2009). Quantification of HCMV transmission is always required due to the unavailability of easy and accurate quantification techniques. Therefore, we established a method to quantify the HCMV transmission and also to phenotypically characterize the HCMV strains based on the mode of transmission.

Most, but not all, fresh clinical HCMV isolates grow strictly by cell-associated transmission in vitro after primary isolation (Wu et al., 2015; Digel et al., 2006). In contrast, laboratory-adapted strains use both modes for their transmission in cell culture due to mutations. The reasons for this difference are not fully understood so far, but might at least partially be related to the viral genes RL13 and UL128 (Dargan et al., 2010; Stanton et al., 2010; Prichard et al., 2001). A classical experiment to study the cell-associated HCMV transmission in cell cultures is the focus expansion assay (Sinzger et al., 1997, 2007; Wu et al., 2015). This assay determines the number of infected cells per focus on different days post-co-culture. However, the focus expansion assay is quite labour intensive, hard to standardize and can only be applied with highly cell-associated viruses (focus formation Fig. 2a). Quantification of virus exhibiting cell-free mode of transmission is not possible using the focus expansion assay.

In our newly established assay (TKA), we first determined the slopes of the linear regression by plotting the percentages of two- or fourfold diluted infecting cells against the percentage of total infected cells at different days post-infection (dpi) quantified by flow cytometer. The slopes were always the same when twofold dilution series was maintained irrespective of the number of starting cells. Therefore, by using the slopes, it was possible to overcome the problem of dependency of the transmission kinetics on the individual number of seeded infecting cells as long as the same dilution series was maintained. The quantification process also became relatively fast, accurate with low inter-test variability (shown from error values).

We used the HCMV Ab as a tool to suppress cell-free transmission, and thus to quantify the relative contribution of cell-free and cell-associated transmission. We showed that the cell-associated transmission occurs in the presence of antibodies in fibroblast cells (Fig. 1b, right) which has also been shown by other groups (Adler et al., 2006; Gerna et al., 2008; Jacob et al., 2013; Fouts et al., 2012). We also showed that the transmission to epithelial cells from infecting fibroblasts was also completely blocked in the presence of antibodies (Fig. 1d, right). We hypothesize that the transmission of infection from HFF to ARPE-19 does only happen by cell-free virus which has been shown for neutralization of HCMV transmission in the presence of HCMV Ab among epithelial cells. Gerna et al. (2008) stated that the neutralizing antibodies are directed against HCMV pUL131A, pUL130 and pUL128 locus. The specific mechanism of virus entry into endothelial/epithelial cells requires the pUL131A-pUL130-pUL128 complex. Neutralizing antibodies against this complex may explain the differences in neutralization between fibroblasts and epithelial cells. Also Fouts et al. (2012) stated that HCMV antibodies against the pentameric gH/gL/UL128/UL130/UL131 complex comprise the majority of the anti-cytomegalovirus neutralizing antibody response. These are the possible explanations for the differential neutralizing activity by the HCMV antibodies seen in our TKA for different cell types.

The TKA was then applied to phenotypically characterize clinical HCMV isolates based on their mode of transmission. By using this approach, we found that a transmission kinetic value of 4.1 (±0.7) was characteristic for a virus showing both cell-associated and cell-free transmission and 0.9 (±0.5), when the virus was mostly transmitted by the cell-associated mode. The results determined by our TKA were verified by using HCMV Ab and by titrating cell-free virus after the application of antibodies to the cells.

Furthermore, we were able to evaluate the effect of different immune interventions in HCMV transmission using TKA in an allogeneic system, providing a readout to quantify the effect of different immune cells in the inhibition of the virus spread in cell culture. First, we demonstrated the effect of IL-2 on NK and T cells in inhibiting the HCMV transmission. NK and T cells both controlled the transmission at a higher rate only after stimulation with IL-2. NK cells do not depend on MHC for their antiviral activity, whereas the T cell effect is dependent on MHC. Therefore, the effect seen with T cells here could be due to MHC incompatibility. The differences detected between NK and T cells concerning the reduction of the HCMV transmission stimulated by IL-2 could also be due to the different amounts of IFN-γ.
secretion. Though IFN-γ is widely known to inhibit HCMV transmission, there could be other cytokines secreted by IL-2 stimulated immune cells which could be responsible for the inhibition of HCMV transmission (De Sanctis et al., 1997; Wu et al., 2015; Jost et al., 2013). We subsequently quantified the effect of NK and T cells on clinical isolates with different modes of transmission. NK cells controlled the transmission more effectively than T cells in both clinical isolates after stimulation with IL-2. Both effector cell types resulted in a more effective inhibition in the clinical isolate exhibiting both modes of transmission than in the clinical isolate exhibiting only the cell-associated mode of transmission. Although several groups described that HCMV specific T cell clones could inhibit HCMV infection using different assays such as CTL assay, and focus expansion assay (Tabi et al., 2001; Sinzger et al., 2007; Jackson et al., 2014), our results using the TKA allow comparative and quantitative determination of the inhibitory effect of T and NK cells. One interesting conclusion from our transmission inhibition experiments is that the immune cells (NK and T cells) could reduce the transmission of clinical isolates irrespective of their mode of transmission (cell-free or cell-associated route). This result is in accordance with the point of view that the immune cells act against infected cells regardless of the route of infection. However, two points have to be considered; (i) accurate quantitative effect of T cells specific for HCMV can be analysed only when using an autologous system and (ii) the clinical isolates we used might have genetic differences, especially viral genes that are important for NK/T immune evasions. The genetic differences of the clinical isolates are under investigation.

Thus, we established a new kinetic assay that is fast, easy to perform and precisely accurate. This robust assay enables us to quantify the HCMV transmission and to phenotypically characterize the HCMV strains based on the modes of transmission. The assay also allows the quantitative determination concerning the contribution of the different modes of transmission in HCMV strains when using neutralizing antibodies. Furthermore, this assay was integrated to screen the quantitative effect of interventions (antibodies, NK cells and T cells) as well as the effect of cytokines on immune cells in inhibiting the HCMV transmission. Therefore, our new assay can be used for any comparative quantitative studies on virus transmission.

**METHODS**

**Ethics statement.** All buffy coat samples were purchased from the Transfusion Center of the Ulm University Hospital (Institut für Klinische Transfusionsmedizin und Immunogenetik Ulm GmbH, Ulm, Germany) and were obtained from healthy HCMV seropositive donors.

**Virus.** TB40/E-delUL16-GFP was generated from TB40/E as described earlier (Digel et al., 2006) and was provided by Professor Dr Christian Sinzger, Institute of Virology, Ulm University Medical Center. Preparation of TB40/E-delUL16-GFP infected HFF stocks: HFFs were infected with virus doses allowing 100% infection rate and frozen at −80°C on 3 dpi. After thawing, the viable cells were counted by using trypan blue to segregate the dead cells. About 90% of the cells resulted viable in

![Graph](image)

**Fig. 4.** Inhibition of HCMV transmission by immune effector cells. The inhibition of transmission by NK cells (with and without IL-2 addition), T cells (with and without IL-2 addition) and HCMV antibodies are shown by plotting the number of newly infected cells per infecting cell against the corresponding days (same presentation as in Fig. 1c, e). The primary graphs as shown in Fig. 1b, d are not shown here. Error bars depict mean values±SD for two different seropositive donors.
every experiment. Preparation of clinical isolates infected HFF stocks: The origin of the clinical isolates E30546, E68240, E56647 and E52812 was described before (Wu et al., 2015). Clinical isolates were initially propagated on HFFs until about 60% of cells showed a cytopathic effect and were used before passage 6 (E30546, E52812 – passage 6; E56647, E68240 – passage 5) in order to better preserve the cells. For preparation of the stocks of cells infected by HCMV clinical isolates, the infected cultures were trypsinized on 3 dpi and frozen at −80 °C for further experiments. The infection rate of the infected cells was determined for every preparation by IEA staining after thawing. Depending on the infection rate, the exact number of infecting cells was seeded in TKA.

Transmission kinetic assay seeding protocol. The number of infecting cells was always determined by IEA staining. Infecting cells were thawed, counted and seeded in twofold dilution from 640 to 10 (640, 320, 160, 80, 40, 20 and 10) or by fourfold dilution from 640 to 40 (640, 160 and 40) along with 20,000 uninfected cells.

Cells. HFF and ARPE-19 were cultured in minimum essential medium (MEM; Gibco) supplemented with 10% fetal calf serum. PBMC were isolated and cryopreserved as previously described (Wu et al., 2013). Thawed PBMC were cultured in minimum essential alpha medium (MEM-α; Gibco) supplemented with 5% human serum (HCMV negative, 56 °C inactivated) and 10% fetal calf serum. NK cells were isolated by negative selection from PBMC using the human NK cell enrichment kit (Miltenyi). Pan-T cells were positively selected from PBMC using T cell isolation kit (Miltenyi). For the co-culture of immune cells with HCMV-infected cells, infected and uninfected cultures were trypsinized on 3 dpi and frozen at −80 °C for further experiments. The infection rate of the infected cells was determined for every preparation by IEA staining after thawing. Depending on the infection rate, the exact number of infecting cells was seeded in TKA.

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Flow cytometric analysis. Live analysis of TB40/E-deUL16-GFP: infected cells were analysed by GFP expression in FACS. The co-cultured cells were trypsinized, washed and treated with propidium iodide (PI) to exclude the dead cells while analyzing in FACS. IEA staining the cells after trypsinization were fixed and permeabilized by 4% PFA and ice cold 80% ethanol. Then the cells were stained for IEA antibodies (pUL122/123, Argene-Biotech) followed by staining with AF488-conjugated goat anti-mouse immunoglobulins (Molecular probes/Invitrogen). Nuclei were counterstained with DAPI.

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IFN-γ detection. Production of IFN-γ was assessed in the co-culture supernatants by commercial ELISA kits for IFN-γ (430104; BioLegend). Cell-free infectivity titration. Supernatants from the day 5 cultures were serially diluted by 10-fold and added to fibroblasts (triplicates) in 96-well plates. After 24 h of co-culture, the cells were fixed and stained for IEA by indirect immunofluorescence (as explained in immunofluorescence section in the materials and methods section).

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REFERENCES


