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Protocols

Enhanced in vitro virus expression using 3-dimensional cell culture spheroids for infection

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ABSTRACT

The way viruses interact with cultured cells and their surrounding environment is still a matter of debate. From a technical point of view, 2D cell cultures only partially exhibit the morpho-molecular pattern required for viral tropism, not reflecting the complexity of the microenvironment *in vivo*. Therefore, 3D cell cultures are envisioned as an alternative approach to study viral replication possibly closer to *in vivo* conditions than 2D, representing the link between traditional cell culture and *in vivo* models.

The use of cellular spheroids is proving to be useful to optimize and overcome constraints related to conventional *in vitro* systems for viral isolation. In order to create an advanced 3D *in vitro* isolation system, we compared the classic 2D shell vial system with the spheroid culture method based on the adhesion inhibition technique with pHema.

In this study, we evaluated which of the most common viral cell lines used in our laboratory (A-549, 293 T, CaCo2, KB, HUH-7, VERO, and MRC-5) (Fig. 1) could be grown as 3D cultures and all proved to be able to grow as spheroids.

Subsequently, we compared the sensitivity and efficiency of isolation of three viral species of medical interest (Adenovirus, CMV, HSV-1) in 2D and 3D cell cultures obtained from the respective susceptible cells. Our results indicate earlier and more sensitive virus isolation than in traditional 2D shell vial system for all three viruses tested, thus confirming how the establishment of 3D culture systems in the virological field is crucial to the improvement and evolution of more accurate and faster virus isolation protocols.

1. Introduction

Spheroids are obtained when cells are cultured in the absence of a surface to adhere to; in these conditions, cells naturally tend to make contact with one another forming a spherical structure made up on a peripheral layer, where cells undergo division, a deeper non-dividing layer and a necrotic core. The environment in spheroids is considered more similar to the one in living tissues than 2-dimensional (2D) cultured cells, because there is no interaction between cells and scaffolding surfaces or with plastic. This is believed to allow cells to preserve crucial interactions with one another that resemble the ones they have in living organisms; in addition, the context allows cells to preserve their tissue-specific phenotype better and may even retain their polarization (He et al., 2016; Laschke and Menger, 2017; Molina-Jimenez et al., 2012; Pampaloni et al., 2007).

Three-dimensional (3D) cultures of cells are used as preclinical models for drug testing and toxicity studies in oncology (Friedrich et al., 2009; Weeber et al., 2017). Very recently, primary human hepatocytes cultured as spheroids were found to express higher levels of proteins involved in drug pharmacokinetics in 3D spheroid cultures. In addition, they turned out to be more temporally stable than in 2D cultures so that they were overall more sensitive to a selection of hepatotoxic compounds (Bell et al., 2018). Methods to analyse the effects of antitumor drugs on tumor cell spheroids were also described: high content image cytometers have been used to measure drug effects on spheroid size and viability with simple stains such as propidium iodide, helping in the screening of drug candidates for drug discovery research.

Animal models have helped elucidate factors that influence viral pathogenesis for many viruses and complex host immune mechanisms. Conventional monolayer cell cultures that support viral replication have also been extremely helpful, but cannot form the complex environment found in animal tissues and, therefore, have a limited

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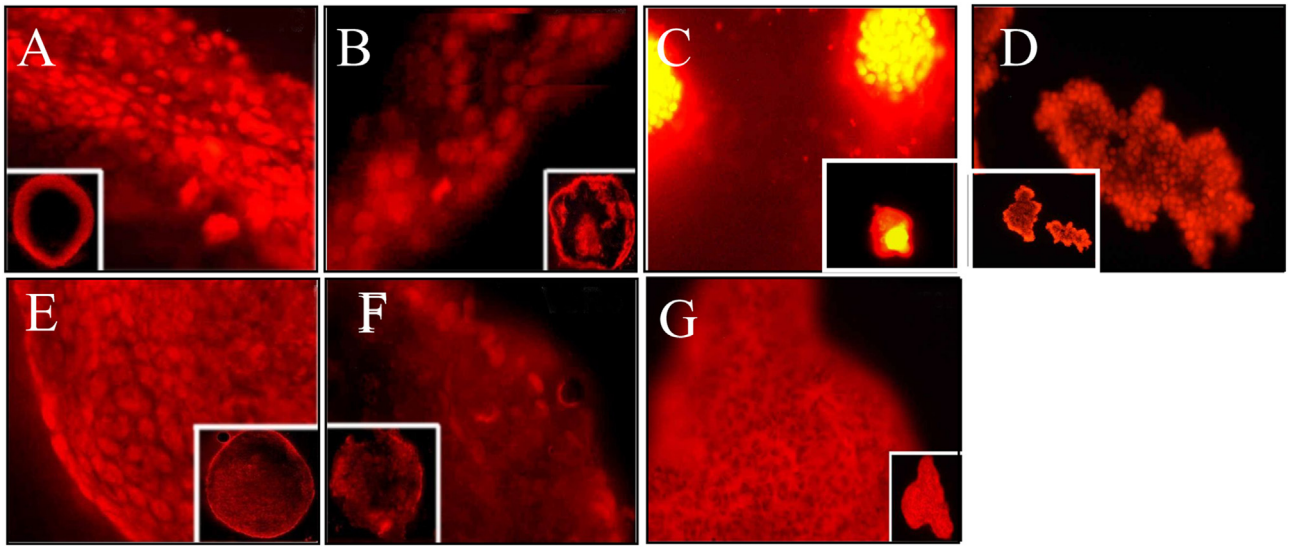

Fig. 1. Spheroids of different cell types. (A) A-549, (B) 293 T, (C) CaCo2, (D) KB (E) HUH-7, (F) VERO, and (G) MRC-5 were cultured by the pHEMA method for 3 days, fixed and stained with PI. Spheroids were visualized by UV light microscopy at 40 × (larger picture) or 20 × (smaller picture enclosed).

translational utility. In addition, viruses that do not infect cultured cells cannot be studied *in vitro* easily. Therefore, 3D cultures are now being tested as models of tissues that can be used to study and diagnose viral infections: 3D HUH-7 cultures were set up and shown to be more polarized and to express higher levels of several receptors and markers and become permissive for hepatitis C virus (HCV) (Sainz et al., 2009; Molina-Jimenez et al., 2012). A more recent study showed that a baculovirus-assisted reovirus infection is able to transduce and kill the normally reovirus-resistant U-118 MG glioma cells in standard cell culture conditions, but U-118 MG tumour cell spheroids were penetrated and killed even more efficiently (Dautzenberg et al., 2017).

A recent study compared culture of four different veterinary viruses in conventional 2D cultures and in 3D rotating wall vessel cultures (Malenovska, 2016), showing that although the yield of virus was lower, the portion of infectious virions produced, as opposed to defective particles, was higher.

Considerable efforts are being devoted in the quest of novel antiviral drugs that can be used against human pathogens and, for this reason, many systems were developed to help improve throughput of increasing numbers of compounds that need to be screened in order to access phase I clinical trials. In this work, we prepared spheroids by two simple methods from several human and primate cell lines. We show that spheroids are obtained with every cell line tested (A-549, 293 T, CaCo2, KB, HUH-7, VERO, and MRC-5).

Spheroids are also shown to be more sensitive to infection by 3 different viruses of medical interest than 2D cultures: herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV) and adenovirus (ADV). Spheroids infected turn positive for viral antigens already at 24 h from high-dose infection. When infected at lower doses of virus, they turn positive for the expression of viral antigen earlier than shell vial cultures.

2. Materials and methods

2.1. Cells and culture conditions

The cells used were: A-549 (human epithelial lung carcinoma cells); VERO (African Green monkey kidney fibroblast cells), CaCo2 (human colorectal adenocarcinoma epithelial cells), HUH-7 (human hepatocellular carcinoma cells), MRC-5 (Human embryonic lung fibroblasts), HEK 293 T (human embryonic kidney cells), and KB (derived from the human HeLa line).

2D cell cultures were maintained in cell culture-grade polystyrene T75 flasks (Sarstedt, Germany) in DMEM supplemented with L-Glutamine, 200 mM (Carlo Erba, Italy), Sodium Pyruvate, 100 mM (Gibco), Penicillin-Streptomycin (Biowest) and 10% Fetal Bovine Serum (Sigma Aldrich). Cells were trypsinized at 70–80% confluency and 50.000 cells were seeded in shell vials (SV); 24 h later, serum was lowered to 2%, and used the next day.

3D cultures were generated by scaffold-free protocols. In the hanging drop method, single-cell suspensions were generated from trypsinized monolayers and diluted to 500,000 cells/ml (Amann et al., 2014). Twenty µl of cell suspension were then dispensed into each of the 96 wells of a microplate (Nunc). Upon inversion of the plate, the hanging drops were held in place by surface tension and cells accumulated at the free liquid–air interface. The plates were incubated under standard conditions.

In the inhibition of cell adhesion method, microplates were coated with poly-2 hydroxyethyl-methacrylate (pHEMA, Sigma Aldrich) (Steadman et al., 2008): pHEMA was dissolved in absolute ethanol (12%); after filtration through a 0.2 µm filter, pHEMA was diluted 10 times in 95% ethanol (Sigma Aldrich). 0.5 ml were put into each well of a 6-well plate and let dry for at least 24 h at RT. At the time of experiment, 100,000 cells in 1 ml of DMEM, 2% FBS, were seeded. After 3–4 days, spheroids could be visualized by eye and used for infection. They were visualized at the UV microscope after fixing and staining in 1 µg/ml propidium iodide, as described below.

2.2. Viruses and infection

Susceptibility to viral infection was tested with primary strains from clinical samples: ADV from a throat swab and HSV-1 from a skin swab, and with prototype laboratory strain CMV AD169.

ADV and HSV-1 were isolated on A549 and VERO, respectively, by the classical shell vial (SV) method (Gleaves et al., 1985). Supernatants from SV were expanded in T25 flasks on the same cells. After 3 days, virus titers were determined by Real time-PCR, as described below, and supernatants were aliquoted for storage at −80 °C.

For infection, VERO, MRC-5 and A549 cells were cultured as spheroids or as 2D cultures for 3–4 days in 1 ml and infected with the viruses listed above. In the sensitivity assay (Fig. 2), SV were added with 10^6, 10^4 or 10^2 copies of each virus in 1 ml, then centrifuged at 800 × *g* at 25 °C for 45 min. SV were then incubated 2 h at 37 °C, then washed once in serum-free medium. One ml of complete culture

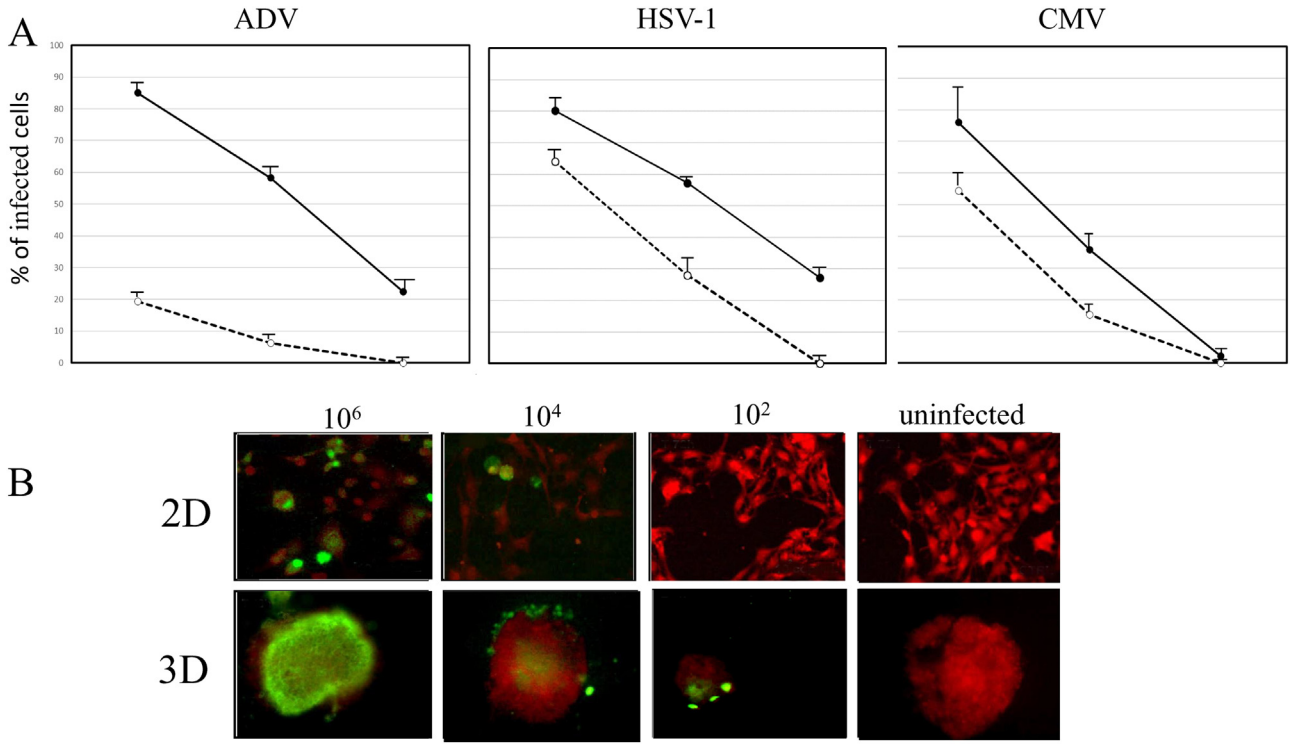

Fig. 2. Sensitivity of 2D cultures or spheroids to infection by different viruses.
(A) 10^6, 10^4, 10^2 copies of ADV, HSV-1, or CMV were used to infect KB, VERO, or MRC-5 cells, respectively, either grown as 2D cultures in SV (○- -○) or as spheroids (●-●), as described in Materials and Methods. Staining with FITC-labelled specific antibodies was carried out 72 h post-infection. Data are reported as mean percentages of infected cells; standard deviations are shown.
(B) VERO cells were cultured as SV cultures (2-D) or as spheroids (3-D) and infected with 10^6, 10^4, 10^2 copies of HSV-1 or left uninfected. Staining with anti- HSV-1 antibody was carried out 72 h post-infection. The typical results of one of the three experiments performed are shown.

medium was then added.

Spheroids were infected by lifting the whole spheroid with a 1,000-µl tip and placing it directly in a pHema-treated well containing 1 ml of the diluted virus. After 2 h at 37 °C, spheroids were removed, washed once in serum-free medium and placed in a new pHema-treated well containing 1 ml complete culture medium.

Cells and spheroids were stained after 72 h from infection with anti-HSV-1/2 fluoresceinated antibody, cat 3293 (Millipore), or anti-CMV fluoresceinated antibody, cat 3245, (Millipore), or anti Adenovirus group fluoresceinated antibody (17-020, Argene, Biomerieux) in solution with Evans' blue, as supplied by manufacturers, for 30 min at 37 °C, then visualized at a UV microscope (Leica) at 20 × and 40 × magnification. The number of fluorescent cells/total cells were counted at the microscope.

In the kinetics experiments (Fig. 3), infection was performed as above, using 10^4 and 10^6 copies of HSV-1, and cells were stained at 24 and 48 h after infection.

2.3. Immunofluorescence

Viral infection was assessed by examining infected cells by direct immunofluorescence on shell vial-cultured cells and in 3D cultures, with spheroids obtained by the pHEMA method. Briefly, at the times from infection specified, cells were washed in PBS and fixed and permeabilized by incubating the SV or spheroids in acetone/methanol, 1:1 for 30 min at −20 °C. Cells were washed in PBS and 100 ul of a solution containing the specific antibodies above, directly labelled with fluorescein, were added. After 30 min at 37 °C, cells were washed and mounted for fluorescence microscopy.

To stain spheroids in Fig. 1, spheroids were washed and fixed as above and incubated 15 min in PBS, 1% propidium iodide (Sigma Aldrich). After washing in PBS, cells were mounted for fluorescence microscopy.

2.4. Real-Time PCR

Viral titers were measured by Real-time PCR on supernatants of infected cells, as described above. Viral nucleic acids were extracted using the QIAsymphony DSP DNA MiVni Kit with the Qiasymphony kit (QIAGEN), following the manufacturers' instructions. Viral loads were determined using the 7500Fast Dx (Applied Biosystem) instrument, using specific commercial kits: ADV R-gene (cod. 69-010, Biomerieux), HSV1-HSV2-VZV R-gene (cod.69-004, Biomerieux) and CMV R-gene (cod.69-003, Biomerieux).

3. Results

3.1. Establishment of spheroid cultures

Two different scaffold-free methods were tested to obtain spheroids from A-549, 293 T, CaCo2, KB, HUH-7, VERO, and MRC-5. In the hanging drop method, a standard number of cells are suspended in a drop of tissue culture medium, where they assemble giving a spheroid. In the pHEMA method, cells are prevented from adhering to a plastic support and grow into a spheroid. Spheroids obtained by this method may have different sizes and derive from the clonal expansion of a single cell and/or from the fusion of several initial spheroids. This method turned out to be easier and less cumbersome, in that it was quite easy for the hanging drop to fall, whereas no specific technical difficulty was found when growing spheroids in pHEMA treated plates. Both methods resulted in spheroids from a number of different cell lines.

A549 and Huh-7 cells grew faster than the others, being visible to the naked eye as round spheroids already after 24 h. KB and Vero cells

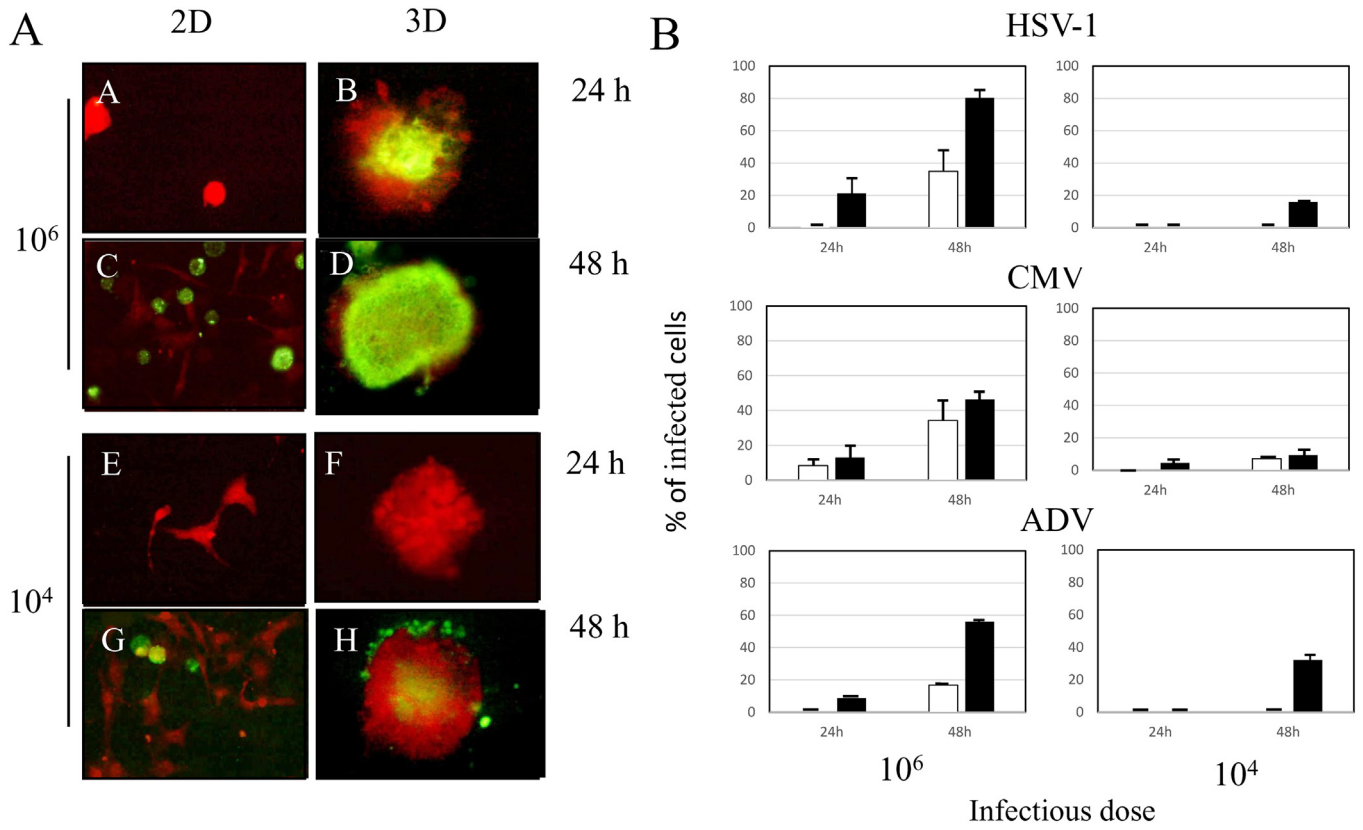

Fig. 3. Kinetics of expression of different viruses using 2D or 3D cultures.
(A) 10^6 and 10^4 copies of HSV-1 were used to infect VERO cells cultured as 2D SV cultures or 3D spheroids. Staining with anti-HSV-1 Ab was carried out 24 or 48 h post-infection and visualized by UV light microscopy at 40 × .
(B) 10^6 and 10^4 copies of the viruses stated above the panels were used to infect VERO (upper panels), MRC-5 (middle panels) or KB cells (lower panels), either grown as 2D cultures in shell vial (empty histograms) or as spheroids (full histograms). Data are reported as mean percentages of infected cells, enumerated at the microscope as described in Materials and Methods; standard deviations are shown. The typical results of one of the three experiments performed are shown.

formed cylinder-shaped 3D cultures; these could be seen less clearly than the previous ones at 24 h. CaCo2 cells formed spheroids the inside of which was made up of smaller, denser cells (Fig. 1C) that stain intensely with propidium iodide, while the outermost cells seemed larger. They could usually be seen by 48 h. The slowest cells to give visible spheroids were MRC5, whose spheroids could only be seen at 72 h. Fig. 1 shows spheroids at day 3 at the light microscope.

Because our aim was to obtain a method to grow spheroids with a reproducible and technically easy method, so that it could be useful in the diagnostic virology laboratory, the following experiments were all performed with spheroids obtained by the pHEMA method.

3.2. Comparison of sensitivity to infection of 2D and 3D cultures

To compare sensitivity to viral infection, spheroids of the relevant permissive cells and SV of the same cell types were infected with a primary strains of ADV, of HSV-1, or with CMV, prototype strain AD169. Viruses were titrated by RT-PCR and 10^6, 10^4 and 10^2 copies/ml were used to infect SV or spheroids. Viral replication was assessed by examining infected cells by direct immunofluorescence after staining for viral antigens with fluorescent antibodies and by evaluating the percentage of infected cells at 72 h post-infection.

As can be seen in the graphs (Fig. 2A), all three viruses turned out to give higher percentages of infected cells in 3D cultures at all quantities used to infect. The most evident effect can be seen with ADV (Fig. 2A left panel), where 10^6 copies gave 3 times as many infected cells in 3D than in 2D cultures. In the middle panel, 100 copies of HSV-1 are already enough to infect 27% of the cells in 3D cultures. Results are less clear, although along the same line, for CMV (Fig. 2A, right panel).

Microscopy results are shown for a typical experiment performed with VERO cells infected with HSV-1 (Fig. 2B). Again, as little as 100 copies of HSV-1 were sufficient to infect 3D cultures. By contrast, no viral antigen could be detected in 2D cultures at 72 h when infected with this amount of virus: even a dose of 10^4 was barely enough to infect cells with HSV-1 in some experiments (such as the one shown in Fig.2 B) but not in others (Fig. 2A, middle panel).

When we tried to determine virus titers in supernatants of these 2D and 3D cultures by molecular methods or in pfu/ml., we obtained increasing amounts of virus at 24 and 48 h of infection in 2D and 3D cultures. However, titers between the 2 types of cultures could not be compared because the cell number making up the different spheroids changed greatly from one experiment to the other.

Thus, 3D spheroid tended to be more sensitive to infection, as determined by immunofluorescence, with the viruses used, although each virus differs in this respect. 3D spheroids could be infected by as little as 100 copies of HSV-1 or ADV.

3.3. Kinetics of viral expression by 2D and 3D cultures

We next assessed the timing of virus expression by spheroids or 2D cultures. Spheroids and SV of the same cell types were infected with 10^4 and 10^6 copies of ADV, HSV-1, and with CMV. Intracellular viral expression was assessed by direct immunofluorescence after staining for viral antigens with fluorescent antibodies and by evaluating the percentage of infected cells at 24 and 48 h post-infection. Fig. 3A shows the results obtained by infecting VERO cells with HSV-1. As can be seen, when cells were fixed at 24 h post-infection, viral protein could only be visualized in spheroids infected at high doses of virus (compare Fig. 3,

panels A and B). At 48 h, 80% of spheroid cells were positive for HSV-1, while roughly half were positive in 2D cultures (Fig. 3A, panels C, D; Fig. 3B upper left panel). Qualitatively similar results were obtained with other cell lines infected with other viruses (Fig. 3B, middle and lower left panels). When 10^4 copies of virus were used, spheroids still proved to have a higher number of infected cells than 2D cultures for HSV-1 and ADV, which was seen best after 48 h post-infection.

Thus, spheroids allow detecting the presence of virus already at 24 h post-infection, roughly 24 h earlier than 2D cultures.

4. Discussion

Viral isolation could be envisioned as the most sensitive method to detect viruses in clinical specimens, because in theory as little as one virion can multiply in appropriate cells and become detectable, given time, even by the least sensitive method. However, many studies demonstrate that various techniques, first of all molecular ones, turn out to be more sensitive than isolation in clinical practice. The development of sensitive and robust 3D culture systems might be a solution to improve sensitivity of viral isolation, and to allow culture of those viruses that are difficult to cultivate with currently used 2D methods. Available 3D biological models include adult and embryonic organ slices and cell cultures in extracellular matrix gels (Pampaloni et al., 2007). These, however, are cumbersome to obtain in the virology laboratory and methods for their maintenance in culture for longer periods of time are lacking at present.

3D cell culture is an emerging technique that allows cells in culture to mimic morphological and biochemical conditions found *in vivo* and is believed to represent normal tissues more closely than the traditional 2D monolayer culture. Spheroids, and 3D cultures in general, are believed to differ from 2D culture in cell polarization and adhesion properties: Baker and Chen (2012) discuss experimental instances where adhesion, migration and polarization, considered to be closer in 3D cultures to what is found in tissues, might be useful.

Spheroids are the easiest 3D cultures that can be obtained and the pHEMA protocol used here can be easily adapted to day-to-day activity, being simple and inexpensive. The culture system proposed may find application in advanced diagnostics and in the evaluation and screening of antiviral drugs, where it would speed up the process, as already shown in other models (Koban et al., 2018). 3D cultures may be potential tools for studying viral infection. In addition, if obtained by labour-saving methods, they may be useful to isolate viruses from clinical samples.

In this study, we were able to obtain spheroids of various size in approximately 3 days with both methods tested without using artificial scaffolding from 7 cell lines (A-549, 293 T, CaCo2, KB, HUH-7, VERO, and MRC-5). We chose to use the pHEMA protocol to obtain spheroids, as it allowed the generation of larger spheroids in a shorter time compared to the hanging drop method (data not shown).

Very few studies address virus infection in 3D cultures. Sainz et al. (2009) showed that 3D culture of liver-derived HUH-7 cells exhibited a more polarized distribution of several surface proteins, e.g. the receptors for HCV, allowing such cultures to become highly permissive for HCV infection. Another study on cervical cancer 3D cultures showed that human papillomavirus receptors were more abundant in such cultures (Ortiz-Sánchez et al., 2016). In the present study, we investigate the properties of spheroids in virus isolation. 2D and 3D cultures of the same cells were compared for sensitivity to viral infection and kinetics of viral expression; infection was carried out with different inocula of viruses and this showed that only spheroids turned out to be positive for viral replication at low doses (10^2 copies/ml, Fig. 2B). Our data also show that 3D cultures, infected by three different viruses of medical interest, are more sensitive to infection than 2D cultures. Spheroids infected with HSV-1 turned out to be positive after only 24 h, if infected with enough virus. This was also true, although not as evident, with a slower growing virus such as CMV (Fig. 2A, left panel).

Kinetics of viral expression was also studied: we show that at high doses of virus inoculum, 3D cultures turned out to be positive for cell staining with virus-specific mAbs already at 24 h post-infection.

To sum up, viral infection turned out to be more efficient in 3D than in 2D cultures, as assessed by enumerating virus-expressing cells. Our data are in line with those reported by Long et al. (1998) and also, much more recently, by Koban et al. who showed that 3D culturing of primary cells increases their sensitivity to infection with cowpox virus (Koban et al., 2018).

Our results apparently disagree with previous results by Molenovska (2016), who showed a lower yield of infectious virions by 3D cultures than by 2D. This, however, might be because 3D cultures only reach a limited size, whereas 2D cultures, if grown in large vessels, may cover considerable areas. This would imply that the number of cells available for viral replication in 3D cultures might turn out to be lower than in 2D. In addition, we only explored viral expression inside cells and not viral release, which may not be proportional. Virus production was determined on supernatants of HSV infected cells (Fig.3A) both by a plaque assay by molecular methods. It increased with time of infection but the yield from spheroids and 2D cultures could not be compared because cell numbers making up spheroids and in SV varied greatly.

The reason why 3D cultures of cells are more susceptible to virus infection might rely on a more abundant density of viral receptor molecules on the surface of 3D-cultured cells. For example, Sainz et al. show that several molecules are more abundant on 3D-cultured HUH-7 than on the same cultured as adherent cells. Some of these molecules were even interpreted as evidence of partial loss of the cancer phenotype of the cells (Sainz et al., 2009). Alternatively, it is also likely that the greater surface area exposure per cell in 3D culture, since no part of the cell is attached to a surface, may also play a role in increased sensitivity.

Detection of virus in 3D cultures may be a sensitive alternative to molecular methods, although the present work did not directly compare molecular and culture sensitivity.

5. Conclusions

This study shows that 3D cultures can be obtained by many different cell lines. The spheroids infected proved to be more sensitive to infection by viruses that traditional cell monolayers. We chose two clinical isolates of ADV and HSV-1, and a laboratory strain, CMV AD169; all appear to infect 3D cells at lower titers that 2D cultures. In addition, expression of viral proteins is faster in 3D cultures. Our findings might be useful in the clinical laboratory, where sensitive methods are required to isolate viruses from clinical samples, and where the speed of response might determine the choice of the virological method used. In addition to a faster diagnosis, easier culture of clinical viral isolates would give an opportunity to study their sensitivity to antiviral agents with biological methods rather than by sequencing.

Further studies to evaluate the breadth of susceptibility to infection with other clinical isolates and viral species are in progress.

Conflict of interest

The authors declare no conflict of interest.

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