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Precision-cut intestinal slices as a culture system to analyze the infection of differentiated intestinal epithelial cells by avian influenza viruses

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A B S T R A C T

Many viruses infect and replicate in their host via the intestinal tract, e.g. many picornaviruses, several coronaviruses and avian influenza viruses of waterfowl. To analyze infection of enterocytes is a challenging task as culture systems for differentiated intestinal epithelial cells are not readily available and often have a life span that is too short for infection studies. Precision-cut intestinal slices (PCIS) from chicken embryos were prepared and shown that the epithelial cells lining the lumen of the intestine are viable for up to 4 days. Using lectin staining, it was demonstrated that α2,3-linked sialic acids, the preferred receptor determinants of avian influenza viruses, are present on the apical side of the epithelial cells. Furthermore, the epithelial cells (at the tips) of the villi were shown to be susceptible to infection by an avian influenza virus of the H9N2 subtype. This culture system will be useful to analyze virus infection of intestinal epithelial cells and it should be applicable also to the intestine of other species.

1. Introduction

The respiratory and the intestinal tract are entry sites that are used frequently by microorganisms to invade a host. In both cases, epithelial cells lining the lumen are a primary target site for successful infection. The intestinal epithelium comprises a variety of cell types that have differentiated into specialized cells to fulfill different functions, e.g. enterocytes, goblet cells, M cells, and others. In addition, non-epithelial cells, such as intraepithelial lymphocytes are present within the epithelial layer. There are no immortalized cell lines that reflect all the functions of well-differentiated epithelial cells. Primary cells are a more promising culture system to analyze the characteristics of these cells. Cultures of primary intestinal epithelial cells are difficult to prepare and often have a limited time of viability that is not sufficient for infection experiments (Bjerknes and Cheng, 1981; Cano-Gauci et al., 1993; Lotz et al., 2006). For physiological studies, the Ussing chamber has proven to be a valuable device to measure the flow of ions across the epithelium (He et al., 2013). The large volume and the constant exchange of the solutions applied, render this system not practicable for infection experiments. Furthermore, the cells are functional only for a limited time.

While infection studies with differentiated intestinal epithelial cells are still a challenging task, there has been progress in recent years in the analysis of respiratory tract infections. Filter-grown airway epithelial cells may differentiate into specialized cells such as ciliated cells or mucus-producing cells, when maintained under air-liquid-interface (ALI) conditions (Slepushkin et al., 2001). This system has been used for human cells and cells from other species (Goris et al., 2009). An alternative culture system for differentiated respiratory epithelial cells is provided by precision-cut lung slices (PCLS), where the epithelial cells are maintained in their original setting (Fisher et al., 1994; AbdEl Rahman et al., 2010; Meng et al., 2013). In addition to mucus production and ciliary activity, this culture system provides an additional feature of the airway,

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bronchoconstriction (Martin et al., 1996). As submucosal cells are also present in precision-cut lung slices, they can be included in the investigation (Goris et al., 2009; Punyadarsaniya et al., 2011).

There is no culture system for differentiated intestinal epithelial cells that is similar to the air-liquid-interface system. However, it has been shown that precision-cut intestinal slices (PCIS) can be used for physiological measurements (De Kanter et al., 2005) providing results that are similar to those obtained with the Ussing chamber (Van de Kerkhof et al., 2006). They have also been used for evaluating drug metabolism in mammals (Groothuis and de Graaf, 2013). This study shows that precision-cut intestinal slices can be used to analyze virus infections.

2. Materials and methods

2.1. Ethics statement

A study approval from an ethics committee was not required, since working with avian embryos is currently not regulated by legislation as animal experiment in Germany (http://www.bmelv.de/SharedDocs/Rechtsgrundlagen/T/Tierschutzgesetz.html), (as confirmed by the animal welfare official of the University of Veterinary Medicine Hannover). Protocols for working with embryonated eggs were in accordance with the European Union Legislation (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32005L0094:EN:NOT). Embryos used for precision cut-intestinal slice preparation did not undergo any procedures prior to being killed by decapitation.

2.2. Precision-cut intestinal slices (PCIS) preparation

Precision-cut intestinal slices were prepared from 20-day-old specific pathogen free (SPF) chicken embryos. This time point was chosen to reduce the risk of contamination from the bacterial microbiota encountered after animals have hatched. The embryos were killed by decapitation. A needle covered with a micropipette tip was inserted carefully into the intestinal lumen to wash away the intestinal content 3 times by phosphate-buffered saline (PBS) pH 7.5 containing NaCl 8.00 g/l (AppliChem, Darmstadt, Germany) KCl 0.20 g/l (AppliChem, Darmstadt, Germany)Na_2HPO_4 1.15 g/l (Merck, Darmstadt, Germany) KH_2PO_4 0.20 g/l (Merck, Darmstadt, Germany)$MgCl_2 \times 6H_2O$ 0.10 g/l (Merck, Darmstadt, Germany) $CaCl_2 \times 2H_2O$ 0.13 g/l (Merck, Darmstadt, Germany). The method used was similar to that described by de Graaf et al. (2010) with slight modifications: (i) for protection of the intestinal tissue, the intestinal contents were removed by washing without applying forceps (ii) a device described for the preparation of precision-cut lung slices (Punyadarsaniya et al., 2011) was used; it can be filled with five pieces of intestine.

The intestine (jejunum) was cut into pieces about 1 inch in length. After having closed one end of the intestine with the help of a thread, the intestine was filled gently with low-melting-point agarose (agarose LM GQT; GERBU, Gaiberg, Germany) to stabilize the intestinal tissue. For solidification, the sample was put on ice. The samples were arranged in a straight position about 1 cm in length and – after removal of the thread by cutting – 5 pieces of intestine were put into the cool tissue holding adapter of a Krumdieck tissue slicer (TSE systems, Bad Homburg, Germany, model MD4000-01). The space between the intestine and the walls of the adapter were filled with low-melting-point agarose. After solidification, the intestine was cut with the Krumdieck tissue slicer into slices approximately 250 μm thick with a cycle speed of 60 slices/min. Precision-cut intestinal slices were incubated in 1 ml of RPMI 1640 medium (Invitrogen/Gibco, Darmstadt, Germany) containing antibiotics and antimycotics; Amphotericin B 2.5 mg/l (Sigma-Aldrich, Munich, Germany), Clotrimazole 1 mg/l (Sigma-Aldrich, Munich, Germany), Enrofloxacin 10 mg/l (Baytril®, Bayer, Leverkusen, Germany), Kanamycin 50 mg/l (Roth, Karlsruh, Germany), Penicillin 0.06 g/l (Sigma-Aldrich, Munich, Germany), Streptomycin 0.05 g/l (Sigma-Aldrich, Munich, Germany) per slice in a 24-well plate at 37 °C and 5% CO_2. The medium was changed every hour during the first 2 h and once after 24 h to remove the agarose before slices were used for infection. The integrity of the cells in precision-cut intestinal slices was determined by applying a Live/Dead viability/cytotoxicity assay kit (Fluo Probes, Rockford, USA, FP-BE4710). For this purpose, the slices were washed with PBS and incubated with calcein AM (1 μM) and ethidium bromide (EthD-1; 2 μM) for 30 min. The former compound is converted into a green-fluorescent dye which is retained by intact cells. Ethidium bromide stains the DNA of cells which have lost the plasma membrane integrity. After incubation, slices were washed with PBS and embedded in Mowiol resin prior to analysis by a Leica TCS SP5 AOBS confocal laser scanning microscope using wavelengths of 488, 561, 405 (excitation) and 570,520, 421 nm (emission) for detection of the green fluorescence of FITC, the red fluorescence of Cy3, and the blue fluorescence of DAPI.

2.3. Virus propagation

An avian influenza virus of the H9N2 subtype was used in this study: A/chicken/Saudi Arabia/CP7/98 (LPAI). It was kindly provided by Hans-Christian Philipp (LohmannTierzucht, Cuxhaven, Germany). Virus stocks were grown in 10-days old specific pathogen-free embryonated chicken eggs (VALO Biomedia, Cuxhaven, Germany). The allantoic cavity of the egg was inoculated with 100 μl of virus solution (virus stock 1:100 in PBS). The inoculated eggs were kept at 37 °C for up to three days in an egg incubator. Chorioallantoic fluid was collected and clarified by low-speed centrifugation (450 × *g*, 15 min). Virus stocks were stored at −80 °C.

2.4. Virus infection

Precision-cut intestinal slices were washed twice with PBS and infected with 500 μl of the viral dilution in RPMI medium. For immunostaining, the slices were infected with 10^5 pfu/ml (1:10 dilution of stock virus) for 2 h. Inoculums were removed and precision-cut intestinal slices were washed 3 times with PBS before a final volume of 1 ml of RPMI medium was added. The slices were incubated in 5% CO_2 at 37 °C for up to 1 day which allowed more than one round of infection. All experiments were performed with at least six slices.

2.5. Preparation of cryosections

Precision-cut intestinal slices were mounted with tissue-freezing medium (Jung, Heidelberg, Germany) on a small filter paper and put into in liquid nitrogen and then stored at −80 °C. Slices were cut by a cryostat (Reichert-Jung, Nußloch, Germany). The sections (10 μm thick) were dried overnight at room temperature and kept frozen at −20 °C until staining.

2.6. Immunofluorescence analysis of cryosections

Samples were fixed with 3% paraformaldehyde for 20 min. The sections were washed 3 times with phosphate-buffered saline minus (PBSM) pH 7.5 containing NaCl 8.00 g/l (AppliChem, Darmstadt, Germany) KCl 0.20 g/l (AppliChem, Darmstadt, Germany) Na_2HPO_4 1.15 g/l (Merck, Darmstadt, Germany) KH_2PO_4 0.20 g/l (Merck, Darmstadt, Germany) followed by permeabilization with 0.2% Triton X-100 for 5 min. A solution of 1% bovine serum albumin was used as diluent for all antibodies that were incubated with the