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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

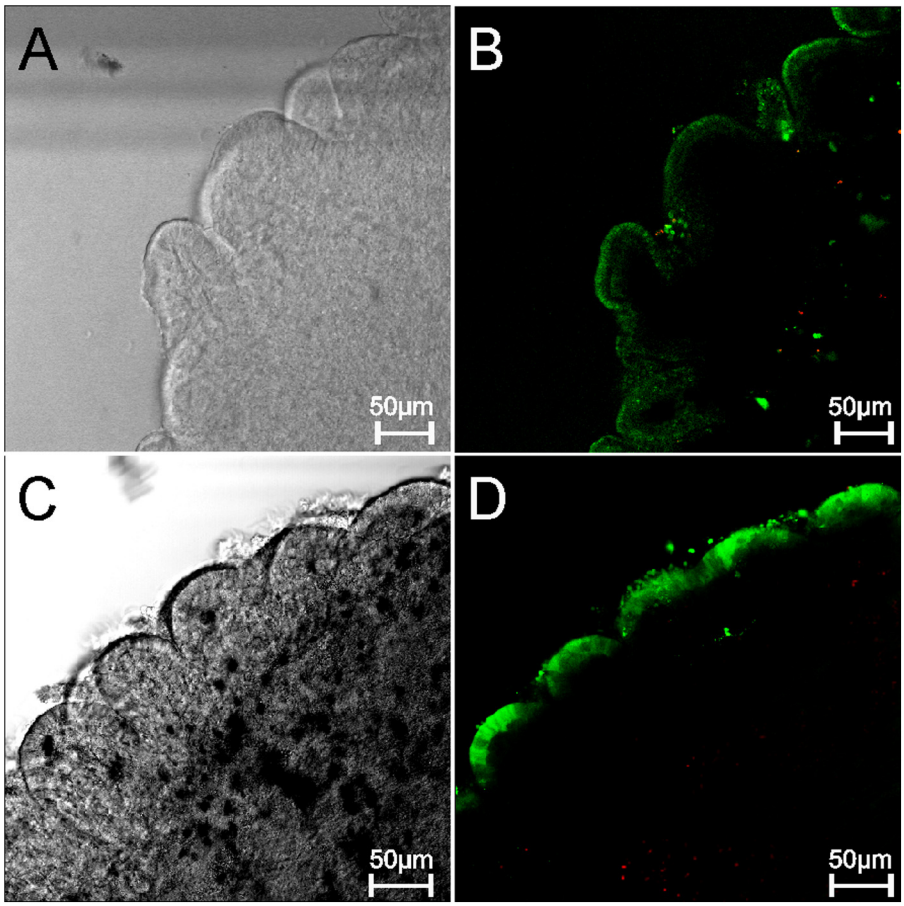

Fig. 1. Characterization by light microscopy of an intestinal slice from day 1 and day 4 after preparation (A and C). Vitality of chicken precision-cut intestinal slices was evaluated by live (green)/dead (red) staining. Slices were stained with a commercial kit at day 1 (B) and 4 after preparation (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sections for 1 h at room temperature in a humid chamber. After the last incubation step, the sections were washed three times with PBS and once with distilled water to remove precipitates that may have been formed during the freezing period. The slices were embedded in Mowiol and stored at 4 °C until examination under the confocal microscope.

A monoclonal antibody against the influenza A virus nucleoprotein (NP) (AbDSeroTec, Düsseldorf, Germany).) at a 1:750 dilution was used for detection of infected cells. The secondary antibody consisted of FITC-labeled anti-mouse IgG (Sigma-Aldrich, Munich, Germany) at a 1:500 dilution.

FITC-labeled *Sambucus nigra* agglutinin (SNA)(Vector laboratories, Burlungame, USA) at a dilution of 1:200 was used to detect α2,6-linked sialic acids and biotinylated *Maackia amurensis* agglutinin II (MAAII) at a dilution of 1:100 was used to visualize α2,3-linked sialic acids after pretreatment with the Avidin/Biotin Blocking kit (both from Vector Laboratories,Burlungame, USA). Streptavidin-Cy3 (Sigma-Aldrich, Munich, Germany) at a dilution of 1:500 was used to visualize binding of biotinylated reagents.

For detection of microvilli, a goat anti-ezrin (Santa Cruz Biotechnology, Heidelberg, Germany) antibody was used at a 1:200 dilution followed by incubation with a Cy3-labeled mouse anti-goat IgG (Sigma-Aldrich, Munich, Germany) as secondary antibody at a dilution of 1:500.

Nuclei of cells were stained by incubation with DAPI (4′,6′-diamidino-2-phenylindole) for 15 min (37 °C).

2.7. Neuraminidase treatment

The precision-cut intestinal slices were washed with RPMI medium 3 times to remove mucus. Washed precision-cut intestinal slices were treated with neuraminidase (NA) type V from *Clostridium perfringens* (Sigma/Aldrich, Munich, Germany) at 50 mU/ring and incubated for 2 h at 37 °C. After removal of the NA solution, precision-cut intestinal slices were washed three times with PBS and then embedded with tissue-freezing medium (Jung, Heidelberg, Germany) to generate cryosections and perform lectin staining.

3. Results

3.1. Precision-cut intestinal slices (PCIS), a model system for differentiated avian intestinal epithelium cells

A culture system was established for differentiated intestinal epithelial cells from the chicken intestine. Precision-cut intestinal slices were prepared from 20 days-old chicken embryos. Precision-cut intestinal slices were analyzed by light microscopy to visualize the morphology of the intestinal slice at day 1 and day 4 after preparation (Fig. 1A and C). To determine whether precision-cut intestinal slices from chicken intestine are a suitable culture system for infection studies, the vitality of the epithelial cells had to be determined.

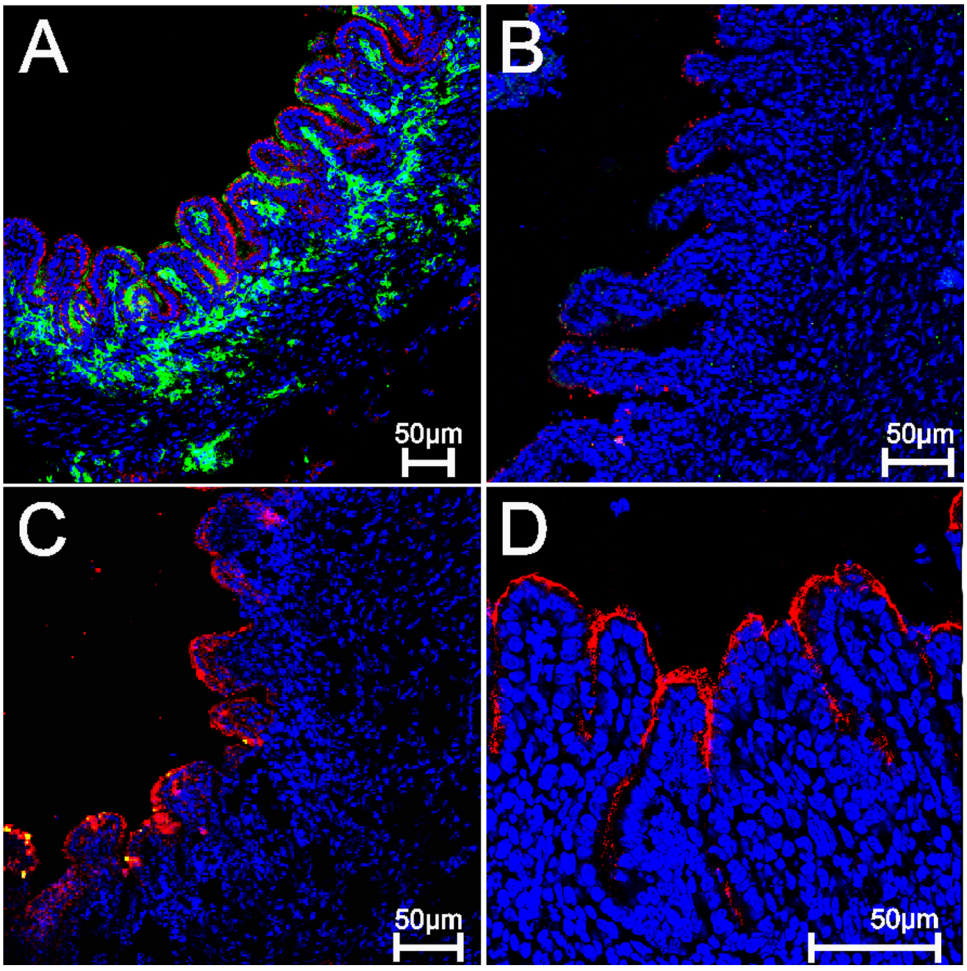

Fig. 2. Cell characterization and infection of chicken PCIS. Cryosections from PCIS were subjected to lectin staining (A): MAA (*Maackia amurensis* agglutinin) staining for α2,3-linked sialic acids (red) and SNA (*Sambucus nigra* agglutinin) staining for α2,6-linked sialic acids (green) or after neuraminidase treatment (B). The type of cells stained in the lamina propria has not been determined. Infection of chicken PCIS by avian influenza viruses H9N2 strain was characterized by immunostaining (C) and the control sample is shown in (D). Cryosections were prepared at 24 h p.i. and used for detection of infected cells and enterocytes. Infected cells were stained with an anti-nucleoprotein antibody (green); enterocytes were stained using an anti-ezrin antibody (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The viability of the cells of precision-cut intestinal slices was analyzed by a live/dead viability/cytotoxicity assay. As shown in Fig. 1, the epithelial lining of the intestine consisted of viable cells (green) when analyzed at 1 and 4 days post-preparation (Fig. 1B and D). The distribution of the red stain indicates that only very few cells of the epithelial lining were dead.

3.2. Expression of sialic acid

Sialic acids are the crucial receptor determinant for the entry of influenza viruses. Therefore, precision-cut intestinal slices were analyzed for the expression of sialic acids on the epithelial cells. Cryosections from precision-cut intestinal slices were stained with sialic acid-specific lectins: *S. nigra* agglutinin (SNA) and *M. amurensis* agglutinin (MAA) typeII. The former lectin recognizes α2,6-linked sialic acids, the latter α2,3-linked sialic acids. As shown in Fig. 2A, the cells stained by the two lectins have a different distribution. Cells stained by SNA (green) were found predominantly in subepithelial regions. This result suggests that α2,6-linked sialic acids are more prominent at the lamina propria that consists of connective tissue. Cells visualized by MAA staining (red) were readily detectable along the epithelial cells lining the intestinal lumen. This finding indicates that α2,3-linked sialic acids are present on the luminal surface of the epithelium. Pretreatment of precision-cut intestinal slices by neuraminidase from *C. perfringens* abolished lectin-specific staining (compare Fig. 2A and B) indicating that binding of both MAAII and SNA was sialic acid-dependent.

3.3. Virus infection of precision-cut intestinal slices (PCIS)

For analysis of the infection of differentiated chicken intestinal epithelial cells, an avian virus of the H9N2 subtype (strain A/chicken/SaudiArabia/CP7/98) was chosen. Most avian viruses have a preference for α2,3-linked sialic acids (Rogers and Paulson, 1983). Avian influenza viruses of the H9N2 subtype have been reported to bind both α2,3- and α2,6-linked sialic acids (Matrosovich et al., 2001; Punyadarsaniya et al., 2011). Strains containing a leucine rather than a glutamine at position 226 of the hemagglutinin, even have a preference for α2,6-linked sialic acids (Matrosovich et al., 2001). The receptor binding activity of the H9N2 virus hemagglutinin used in this study is able to bind to both α2,3- and α2,6-linked sialic acids as indicated by glycan array analysis (Punyadarsaniya et al., 2011).

To detect infected cells, cryosections of chicken precision-cut intestinal slices were stained for the presence of viral antigen using an antibody directed against the nucleoprotein (Fig. 2C, green). For comparison, the cryosections were stained in parallel for the presence of ezrin at the surface of the epithelium (Fig. 2C, red). The

infection by H9N2 virus was limited to the epithelial cells lining the intestinal lumen. Co-staining of the cryosections for the presence of viral antigen and ezrin revealed that the virus was able to infect villous cells (Fig. 2C). The uninfected precision-cut intestinal slices were used as a control (Fig. 2D). Virus released into the supernatant was determined by plaque titration and found to increase from 10^3 pfu/ml at 24 hpi to 10^4 pfu/ml at 48 hpi.

4. Discussion

In this study, a culture system for chicken intestinal epithelial cells was introduced that is suitable for infection studies. In previous reports, different approaches have been applied to analyze the infection of intestinal epithelial cells (IEC). In contrast to respiratory epithelial cells, IEC cultures are more difficult to establish. Thus, the culture systems introduced so far have all some shortcomings, e.g. a short life span, or the selection of only some specialized cells such as crypt cells (Bjerknes and Cheng, 1981; Cano-Gauci et al., 1993; Lotz et al., 2006). Precision-cut intestinal slices are an interesting alternative as their preparation is comparably easy providing a large number of slices. Furthermore, the epithelial cells lining the intestinal lumen remain viable for up to 4 days, a life span long enough for virus infections. The feasibility of this approach has been demonstrated with the avian influenza virus of the H9N2 subtype. While influenza virus infections in many cases are restricted to the respiratory tract, infection of Mallard duck by influenza viruses is a typical lower intestinal infection (França et al., 2012). Therefore, it will be interesting to use precision-cut intestinal slices from duck intestines for infection studies. In this study, the infection system was established for the chicken intestine, because the chicken is used routinely in the laboratory tests, e.g. for preparation of chicken embryo kidney cells. Future work will include the preparation of precision-cut intestinal slices from other avian species. The preparation method for precision-cut intestinal slices reported in this study is limited by the organ size. It is expected that this technique can also be applied to the intestinal tissue from small mammalian species e.g. from mice and bats. Thus, precision-cut intestinal slices promise to be a valuable culture system for infection studies with enteric viruses.

Author contributions

Conceived and designed the experiments: DP, GH; Performed the experiments: DP; Analyzed the data: DP, GH, CW, AKW, SR, MA, HN; Contributed reagents/materials/analysis tools: CW, AKW, SR, MA, HN; Wrote the paper: DP,GH.

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