ScienceDirect

ELSEVIER

Journal of Virological Methods 142 (2007) 105–112

Virological Methods

www.elsevier.com/locate/jviromet

In vitro culture of porcine respiratory nasal mucosa explants for studying the interaction of porcine viruses with the respiratory tract

Sarah Glorieux [a], W. Van den Broeck [b], K.M. van der Meulen [a], K. Van Reeth [a], H.W. Favoreel [a,c], H.J. Nauwynck [a,*]

[a] *Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium*
[b] *Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium*
[c] *Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium*

Received 21 August 2006; received in revised form 18 December 2006; accepted 18 January 2007
Available online 26 February 2007

Abstract

The mucosal surface of the respiratory tract is a common site of entry of many viruses. Molecular and cellular aspects of the interactions of respiratory viruses with the respiratory nasal mucosa are largely unknown. In order to be able to study those interactions in depth, an in vitro model was set up. This model consists of porcine respiratory nasal mucosa explants, cultured at an air–liquid interface. Light microscopy, scanning electron microscopy and transmission electron microscopy, combined with morphometric analysis and a fluorescent Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL) staining were used to evaluate the effects of in vitro culture on the integrity and viability of the explants. The explants were maintained in culture for up to 60 h post-sampling without significant morphometric (epithelial thickness, epithelial morphology, thickness of the lamina reticularis, continuity of the lamina densa, relative amounts of collagen and nuclei) changes and changes in viability. The potential to infect the explants was demonstrated for two porcine respiratory viruses of major importance: suid herpesvirus 1 and swine influenza virus H1N1. In conclusion, this in vitro model represents an ideal tool to study interactions between infectious agents and porcine respiratory nasal mucosa.

Keywords: In vitro model; Respiratory nasal mucosa explants; Morphometry; Viability; Respiratory virus

1. Introduction

The mucosal surface of the respiratory tract is a common site of entry of many viruses. A respiratory virus enters the nasal cavity, makes contact with the mucosal surface and infects mucosal epithelial cells. Most viruses stop here; some viruses cross the basement membrane and infect the underlying connective tissue. The different aspects of viral replication in the mucosal epithelial cells and the invasion of viruses through the basement membrane, which forms a barrier, are largely unknown.

Suid herpesvirus 1 and swine influenza virus are two important porcine respiratory viruses with different pathogenicity. With suid herpesvirus 1 (SHV1, also called pseudorabies or Aujeszky's disease virus), a model virus for alphaherpesviruses, infection starts at the mucosal surface of the nasal cavity, nasopharynx, trachea and lungs (Wittmann et al., 1982). Most alphaherpesviruses have accumulated during their evolution interesting tools to allow penetration through the basement membrane and spread in the underlying lamina propria (Appel et al., 1969; Gibson et al., 1992; Maeda et al., 1998; Nauwynck, 1997; Wyler et al., 1989). By doing so, SHV1 reaches blood vessels and nerve endings in the underlying lamina propria and spreads throughout the body (Wittmann et al., 1980). A crucial step in invasion of SHV1 is the crossing of the basement membrane. The mechanism of penetration through the basement membrane is largely unknown. An in vitro model would be useful in order to elucidate this invasion mechanism. In contrast, infection with type A swine influenza viruses (SIVs) is generally limited to respiratory epithelial cells (Van Reeth and Nauwynck, 2000). The viral replication cycle in the target cell has been poorly studied and, therefore, a physiologically

* Corresponding author. Tel.: +32 9 264 73 73; fax: +32 9 264 74 95.
E-mail addresses: sarah.glorieux@UGent.be (S. Glorieux), wim.vandenbroeck@UGent.be (W. Van den Broeck), karen.vandermeulen@UGent.be (K.M. van der Meulen), kristien.vanreeth@UGent.be (K. Van Reeth), herman.favoreel@UGent.be (H.W. Favoreel), hans.nauwynck@UGent.be (H.J. Nauwynck).

0166-0934/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2007.01.018

relevant model that resembles closely the in vivo situation is needed.

An in vitro model was established in the present study. Porcine nasal respiratory mucosal explants were maintained at an air–liquid interface in in vitro culture for up to 60 h post-sampling. Light microscopy, scanning electron microscopy, transmission electron microscopy and fluorescence microscopy were used to evaluate the effects of in vitro culture on the morphometry and viability of the explants. Further, the utility of the explants for studying aspects of viral replication in the respiratory tract was tested by assessing their susceptibility to SHV1 and SIV (H1N1). More knowledge about pathogenic aspects of viral replication and invasion in nasal mucosa may contribute to the development of more efficient and effective therapeutic and preventive strategies.

2. Materials and methods

2.1. Animals

Three 4-weeks-old piglets from a high health status farm were housed together in an experimental unit with free access to food and water. For 3 d, the piglets were treated daily intramuscularly with antibiotics (6.25 mg/kg body weight enrofloxacin (Baytril®, Bayer) and 6.25 mg/kg body weight lincomycin with 12.5 mg/kg body weight spectinomycin (Linco-Spectin®, Pfizer)).

2.2. Isolation and cultivation of the respiratory nasal explants

The piglets were euthanised at 6–8 weeks old. After euthanasia with 12.5 mg/kg body weight thiopental (Pentothal®, Kela), the piglets were exsanguinated. The head was removed from the carcass and the nose was sawed off the skull at a level just in front of the eyes. Nasal explants were carefully stripped from the surfaces of the ventral turbinates and septum using surgical blades (Swann-Morton). Pieces of respiratory mucosa 16 mm^2 in size were cut and placed with the epithelial surface upwards on fine-meshed gauze for culture at an air–liquid interface. Serum-free medium (50% RPMI (Gibco)/50% DMEM (Gibco)) supplemented with 0.3 mg/ml glutamine (BDH Biochemical), 1 μg/ml gentamycin (Gibco), 0.1 mg/ml streptomycin (Certa) and 100 U/ml penicillin (Continental Pharma)) was added until just a thin film of fluid covered the epithelium. During the cultivation period, medium was not replaced. Ciliary beating was checked on a daily base.

2.3. Morphometric analysis

At 0, 24 and 60 h of cultivation, explants were taken for morphometric analysis.

2.3.1. Light microscopy

Explants were fixed in a phosphate-buffered 3.5% formaldehyde solution for 24 h. After fixation, the samples were embedded in paraffin in an automated system (Shandon Citadel Tissue Processor, Cheshire, UK). Sections 8 μm thick were made, deparaffinised in xylene, rehydrated in descending grades of alcohol, stained, dehydrated in ascending grades of alcohol and xylene and mounted with DPX (DPX mountant, BDH Laboratory Supplies, Poole, UK).

Haematoxylin–eosin staining was used to estimate the epithelial thickness. Using Soft Imaging System GmbH analySIS® software (Olympus, Germany), the effect of in vitro culture of nasal respiratory explants on the epithelial morphometry was evaluated by measuring the epithelial thickness (Fig. 1a–c). For each sample, the thickness was measured at five randomly selected places in five random fields.

Reticulin staining was performed to visualise the collagen type III reticular fibers of the lamina reticularis of the basement membrane (Bradbury and Gordon, 1977a). Using this stain and the Soft Imaging System analySIS®, the thickness of the lamina reticularis was evaluated (Fig. 1d–f). The thickness was measured at five randomly selected places in five random fields of each sample.

Van Gieson staining marks all types of collagen, especially collagen type I (Bradbury and Gordon, 1977b). The integrity of the lamina propria was analyzed using this stain. Using Soft Imaging System analySIS®, the relative amounts of collagen and nuclei in a defined region of interest (roi) in five randomly selected fields of each sample were calculated by setting a threshold (Fig. 1g–i).

All stained sections were analyzed with an Olympus BX61 light microscope at 40× magnification.

2.3.2. Scanning electron microscopy

The explants were fixed in HEPES-buffer with 2.5% glutaraldehyde for 24 h. The samples were post-fixed in 1% osmium tetroxide for 2 h at room temperature. In the next step, the fixed tissue samples were dehydrated through ascending grades of alcohol and transferred to a critical point drier CPD 030 (Baltec, Balzers, Liechtenstein). The dried tissues were mounted on a metal stub and sputter-coated with platinum. Examination of the samples was performed on a JEOL JSM 5600 LV scanning electron microscope (JEOL Ltd., Tokyo, Japan). Using 3000× magnification, the different cell types were counted. Ciliated cells contain numerous cilia on their apical surface. Non-ciliated cells comprise different cell types: brush cells, goblet cells and another type of secretory cells. Brush cells are non-secretory cells bearing apical microvilli. Goblet cells are secretory mucous cells. Another type of secretory cell, which contains few microvilli with thin hairlike projections on its apical surface, has also been described (Martineau-Doize and Caya, 1996). Indeterminate cells, which could not be assigned to one of the three types of non-ciliated cells, were classified as 'other'. For each sample, five fields of 40 cells were counted.

2.3.3. Transmission electron microscopy

Tissue samples were incubated overnight at 4 °C in Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4)) (Karnovsky, 1965). The samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) for 8 h, after which they underwent

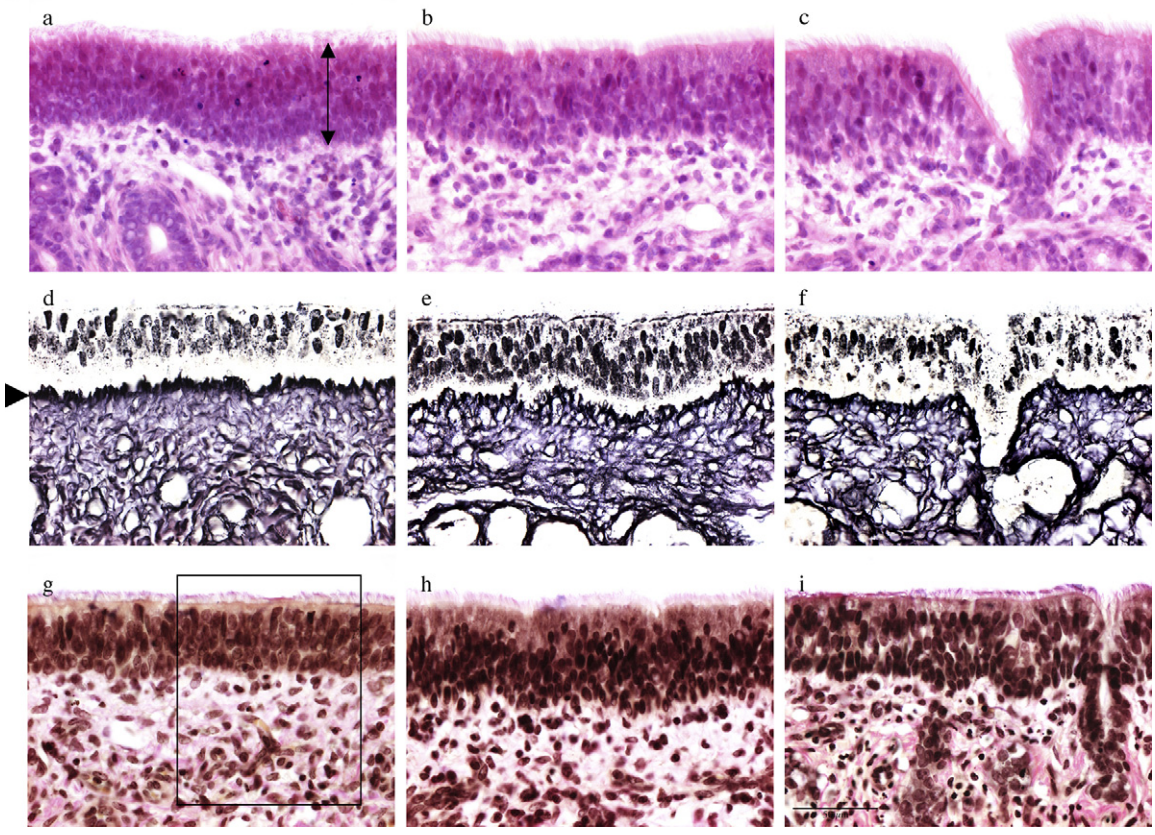

Fig. 1. Light photomicrographs of porcine respiratory nasal explants at 0, 24 and 60 h of in vitro cultivation. Eight-micron-sections were stained with haematoxylin–eosin (a–c) for evaluation of the epithelial thickness (indicated by arrow). A reticulin staining (d–f) was performed to measure the thickness of the lamina reticularis (indicated by arrowhead). A van Gieson stain (g–i) was used to count the relative amounts of collagen and nuclei within a region of interest (roi indicated by a rectangle). By setting a threshold, different colors were assigned to collagen and nuclei, respectively, and the percentages of collagen and nuclei were counted within this roi.

an overnight post-fixation procedure in 2% osmium tetroxide at 4 °C. Each of the previous steps involved rotary motion of the solution during the incubation period. Dehydration was performed through a series of graded alcohols. The dehydrated samples were infiltrated with a low viscosity embedding (LVR) medium (Agar Scientific, Stansted, UK) for 2 d and then embedded in LVR. Ultrathin sections were cut using a glass knife on an Ultramicrotome Ultracut S (Leica, Vienna, Austria). Sections were stained with a Leica EM stain and examined on a JEM-1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at 60 kV.

2.4. Analysis of viability

2.4.1. Immunofluorescence microscopy

DNA fragmentation was detected using the In Situ Cell Death Detection Kit (Fluorescein) obtained from Roche (Mannheim, Germany), which is based on Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL). The TUNEL reaction was performed according to the manufacturer's instruction. The samples were analyzed with a fluorescence microscope (Leica DM RBE microscope, Leica Microsystems GmbH, Heidelberg, Germany). TUNEL-positive cells were counted in the epithelium as well as in the lamina propria. For both the epithelium and the lamina propria, five randomly selected fields of 100 cells were evaluated.

2.5. Statistical analysis

The data were statistically evaluated by analysis of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, Illinois, USA). Results that had P-values of ≤ 0.05 were considered significant. The data shown represent means + S.D. of triplicate independent experiments.

2.6. Virus, inoculation and evaluation of virus replication

SHV1 (89V87) (Nauwynck and Pensaert, 1992) and swine influenza virus H1N1 (A/Sw/Belgium/1/98) (Van Reeth et al., 2004) were used.

The explants were kept in culture for 10 h before inoculation. Explants were taken from their gauze, placed in a 24-well plate with the epithelial surface upwards and washed twice with warm medium. Afterwards, they were inoculated with 600 μl inoculum containing 3×10^6 $TCID_{50}$ of SHV1 (89V87) and 10^6 $TCID_{50}$ of influenza virus H1N1 (A/Sw/Belgium/1/98). After incubation for 1h at 37 °C and 5% CO_2, explants were washed three times with warm medium and transferred again to the gauzes.

At different timepoints post-inoculation (pi), the explants were embedded in methocel® (Fluka), frozen at −70 °C, cryosectioned, fixed in methanol (−20 °C, 100%) and stained. For staining of the SHV1-infected explants, explants were incubated with FITC-labeled porcine polyclonal anti-SHV1 antibodies (Nauwynck and Pensaert, 1995). H1N1 (A/Sw/Belgium/1/98)-infected explants were stained using mouse monoclonal antibodies against influenza virus A nucleoprotein (ATCC number HB-65) followed by FITC-labeled goat anti-mouse antibodies (Molecular Probes, Eugene, USA). Finally, cryosections were washed three times in wPBS and mounted with glycerin-DABCO. The staining was investigated with a confocal microscope (Leica TCS SP2 confocal microscope, Leica Microsystems GmbH, Heidelberg, Germany). An argon 488 nm laser was used to excite FITC-fluorochromes.

3. Results

3.1. Ciliary beating

The cilia of the epithelial cells continued beating for at least 60 h after sampling.

3.2. Viability of the tissue explants

The effect of cultivation time on the viability of the explants is shown in Table 1. No significant increase in the number of TUNEL-positive cells in the epithelium or lamina propria was found with increasing time post-sampling.

3.3. Epithelial morphometry

3.3.1. Light microscopy

The respiratory epithelium showed no significant changes in epithelial thickness during in vitro culture (Figs. 1a–c and 2a).

3.3.2. Scanning electron microscopy

Using scanning electron microscopy, ciliated and non-ciliated cells were distinguished. Representative scanning electron micrographs of explants at 0, 24 and 60 h of in vitro culture illustrate ciliary cells and the different types of non-ciliary cells (Fig. 3). The relative percentages of ciliary and non-ciliary cells are shown in Fig. 4a and b illustrates the measured percentages of the different types of non-ciliary cells. With increasing time of in vitro culture, no significant changes in the percentages of ciliary and non-ciliary cells were observed.

Table 1
Occurrence of apoptosis in the epithelium and lamina propria in porcine nasal explants during in vitro cultivation

	%TUNEL-positive cells at . . . h of cultivation		
	0	24	60
Epithelium	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2
Lamina propria	0.7 ± 0.6	3.5 ± 2.9	4.5 ± 3.2

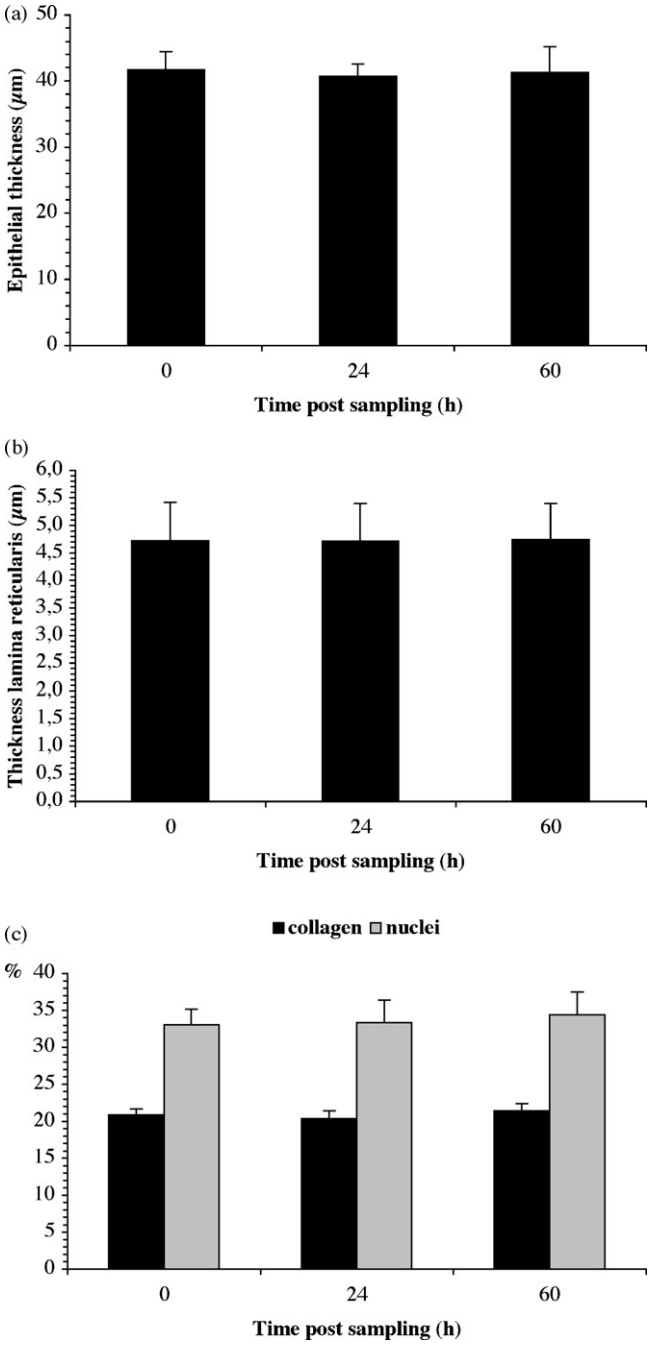

Fig. 2. Epithelial thickness (a), thickness of the lamina reticularis (b) and percentages of collagen and nuclei within a region of interest (c) in explants at different time points during cultivation. Data are represented as means + S.D. (error bars).

Also, the percentages within the different types of non-ciliated cells after 0, 24 and 60 h of cultivation did not change significantly.

3.3.3. Transmission electron microscopy

Using transmission electron microscopy, different epithelial cell–cell contacts were distinguished between basal cells in comparison with apical cells at 0 h after sampling. Basal epithelial cells were separated by intercellular spaces. In contrast, apical

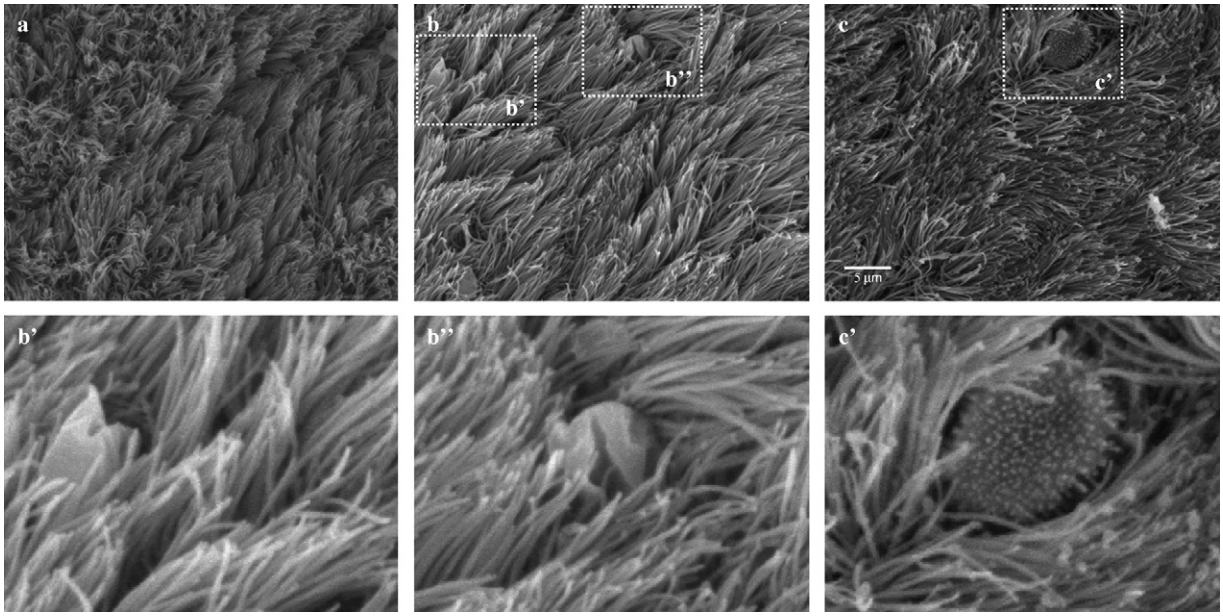

Fig. 3. Scanning electron micrographs of porcine respiratory nasal epithelium at 0 (a), 24 (b) and 60 h (c) of in vitro cultivation. (b′ and b″) Shows a goblet cell, (c′) illustrates a brush cell.

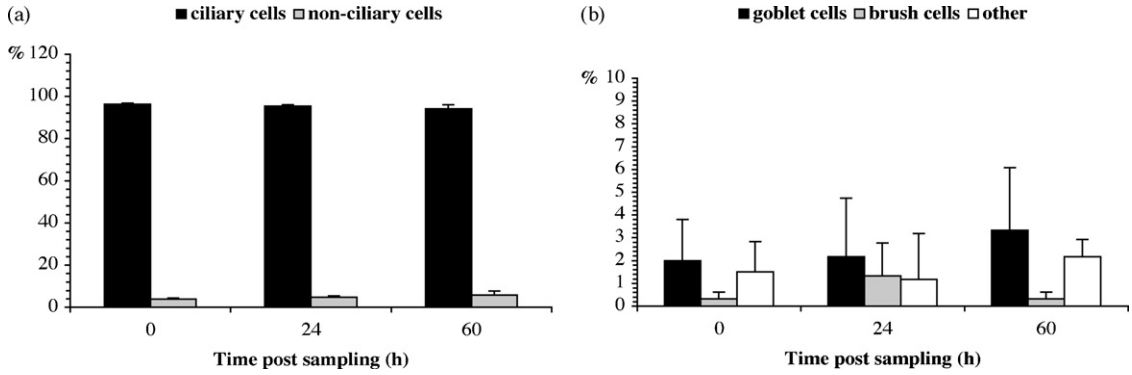

Fig. 4. Percentage ciliary and non-ciliary cells at 0, 24 and 60 h after sampling (a), percentage of different non-ciliary cell types: goblet cells, brush cells and 'other' at 0, 24 and 60 h after sampling (b). The data are represented as means + S.D. (error bars).

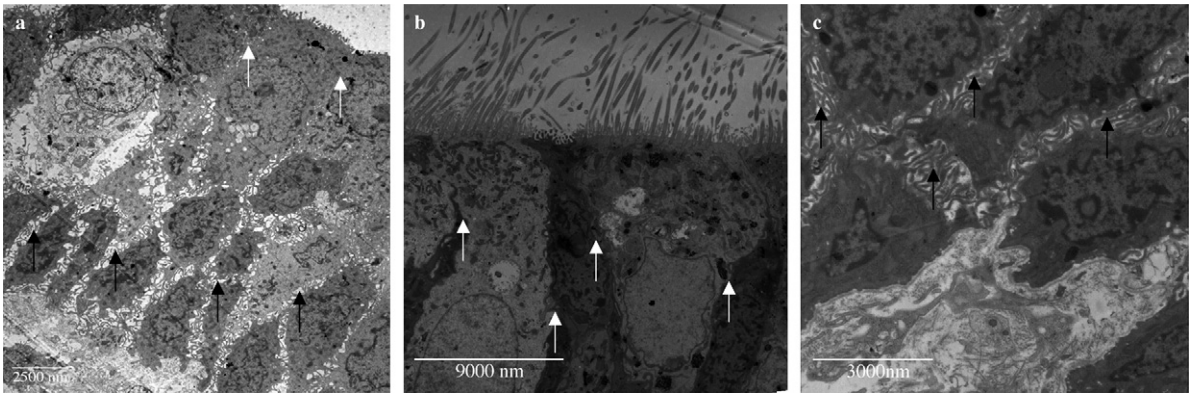

Fig. 5. Representative transmission electron micrographs of the close cell–cell contacts between apical cells (white arrows) and the intercellular spaces between basal cells (black arrows). (a) Represents an overview of porcine nasal epithelium, (b and c) illustrate apical cells and basal cells, respectively.

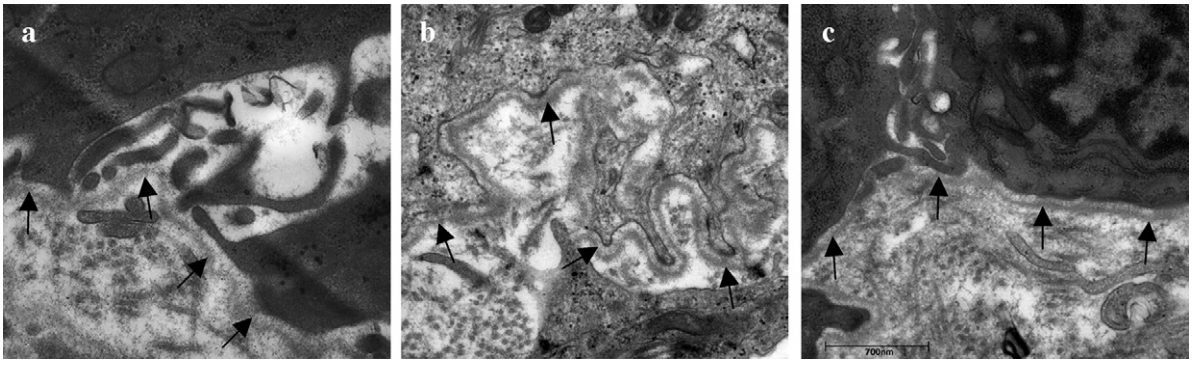

Fig. 6. Transmission electron micrographs of porcine respiratory nasal explants at 0 (a), 24 (b) and 60 h (c) of in vitro cultivation. Arrows indicate the lamina densa between the epithelium and underlying lamina propria.

cells were adjacent. A similar pattern was seen at 24 and 60 h after sampling. However, it should be remarked that at 60 h after sampling rare small intercellular spaces between the apical cells were also seen. Transmission electron microscopic images of the epithelial cellular contacts are shown in Fig. 5.

3.4. Basement membrane morphometry

3.4.1. Light microscopy

Throughout 60 h of in vitro culture, no significant changes were observed in the thickness of the lamina reticularis (Figs. 1d–f and 2b).

3.4.2. Transmission electron microscopy

Using transmission electron microscopy, the continuity of the lamina densa at 0, 24 and 60 h after sampling was visualised (Fig. 6). A variance in thickness could be observed at each time point post-sampling.

3.5. Morphometry of the lamina propria and overlying epithelium

The morphometry of the lamina propria and overlying epithelium was evaluated using van Gieson staining. With increasing time after sampling, no significant changes were observed in the relative amounts of collagen and nuclei (Figs. 1g–i and 2c).

3.6. Evaluation of viral primary replication

Using confocal microscopy, infected cells were observed for SHV1 (89V87) and swine influenza virus H1N1 (A/Sw/Belgium/1/98) (Fig. 7). Fig. 7a and b shows repre-

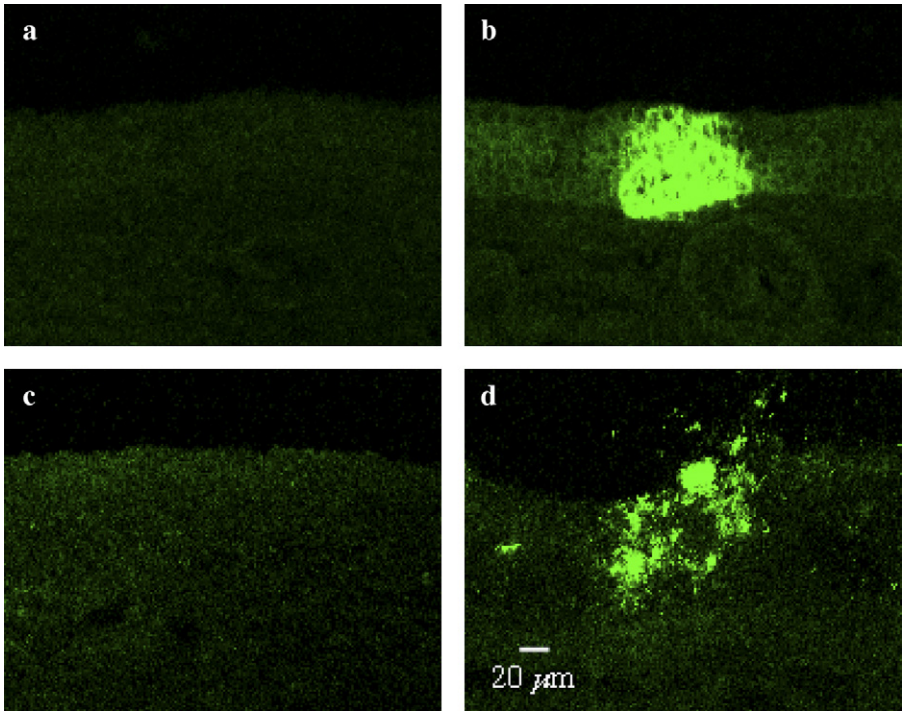

Fig. 7. Confocal microscopic images of SHV1-infected porcine respiratory nasal explants at 0 (a) and 24 h (b) pi and of swine influenza virus H1N1 (A/Sw/Belgium/1/98)-infected cells in explants at 0 (c) and 48 h (d) pi.

sentative fluorescence micrographs of SHV1-infected porcine respiratory nasal explants at 0 and 24 h pi, respectively. Fig. 7c and d illustrates H1N1 (A/Sw/Belgium/1/98) infection at 0 and 48 h pi, respectively.

4. Discussion

In the present study, an in vitro porcine explant system was set up to study the replication of viruses in porcine respiratory nasal mucosa.

Human and rat nasal explant culture systems have been described previously. Human nasal mucosal explants have been cultured at an air–liquid interface for up to 48 h using a gelatin sponge surrounded by medium (Schierhorn et al., 1995); for up to 6 d on plastic plates, placed on a rocking platform (Ali et al., 1996); for up to 20 d with daily replacement of the medium and for up to 4 d without medium replacement using a strip of filter paper with the two ends immersed in MEM (Jackson et al., 1996); for up to 7 d using a prehydrated gelfoam (Jang et al., 2005). Rat nasal airway tissue has been cultured on mesh inserts for up to 14 d at an air–liquid interface (Fanucchi et al., 1999). The cultivation of porcine nasal explants at an air–liquid interface with daily replacement of the medium was used by Pol (1990). However, a morphometric analysis and viability evaluation were not performed.

In the present porcine model, porcine nasal respiratory explants, dissected from the nasal septum and the ventral conchae of 6–8-week-old piglets, were cultured at an air–liquid interface on fine-meshed gauze for up to 60 h without replacement of the medium. Cultivation at an air–liquid interface on fine-meshed gauze with medium on one side of the tissue and air on the other creates a physiologically relevant environment (Johnson et al., 1993; Whitcutt et al., 1988) and is a common feature of the cultivation techniques described above. In the present porcine model, cultivation was performed in serum-free medium. Serum-free conditions were also used for the cultivation methods described above, except for the method described by Pol (1990). The use of fetal calf serum in the present nasal explant model (data not shown) resulted in an epithelium with reduced integrity: epithelial cells became enlarged, cell–cell contacts were lost and the epithelium became loose. An increase in the cultivation period of epithelial cells by replacement of serum-supplemented medium by serum-free medium has been described by Gruenert et al. (1990).

In contrast to cells in nasal epithelial cell culture systems (Lin et al., 2001; Lopez-Souza et al., 2003; Schmidt et al., 1996; Yoon et al., 2002), cells in explants maintain their morphology. Using tissue explants, normal cell–cell contacts, and consequently, the three-dimensional structure of the tissue, are retained. Therefore, explants are ideal to mimic the in vivo situation.

In the present study, it was demonstrated that porcine respiratory nasal explants could be maintained in culture on fine-meshed gauzes at an air–liquid interface with serum-free medium for at least 60 h. Ciliary beating was maintained during this cultivation period. There were no significant effects of in vitro cultivation on the morphometry and viability of the explants. The epithelial thickness and morphology, the thickness of the lamina reticularis and the relative amounts of collagen and nuclei remained similar throughout the period of cultivation. Transmission electron microscopy was used to visualise the basement membrane and the integrity of the porcine nasal respiratory epithelium. The lamina lucida and lamina densa could be distinguished. At 0, 24 and 60 h after sampling the lamina densa showed a similar pattern of a continuous lining. At each time point, a variability in the thickness of the lamina densa could be observed. Possible explanations are a different contact with a certain cell type, or a local alteration in composition due to a local imbalance between degradation and formation. Transmission electron micrographs of the respiratory epithelium at 0 h after sampling revealed that basal epithelial cells showed intercellular spaces, in contrast to the apical epithelial cells, which had close cell–cell contacts. This pattern was maintained until 60 h after sampling. At that time point, some explants showed a few small intercellular spaces between the apical epithelial cells. This could be due to a decreased strength of the cell–cell contact at these sites. Finally, there was no significant increase in the number of apoptotic cells during the cultivation period. We can conclude that the tissue explants retained their integrity and viability throughout the experimental period.

Infection of porcine respiratory nasal explants was demonstrated for SHV1 (89V87). Thus, respiratory explants could be useful for investigation of the SHV1 invasion mechanism. Primary replication of swine influenza virus H1N1 (A/Sw/Belgium/1/98) in porcine nasal respiratory explants was also demonstrated. Therefore, the present in vitro model is a useful tool for investigation of cellular and molecular interactions of infectious agents with the nasal mucosa.

The use of an explant model reduces the number of experimental animals required. Twenty-four explants can be obtained from one pig. This results in a reduction of the number of experimental animals by a factor of 24. An in vitro explant model also replaces in vivo pig experiments in which animals are exposed to viruses and become diseased. Therefore, the in vitro model satisfies the principles of the 3 Rs of Russell and Burch (1959), which include reduction, refinement and replacement. Reduction consists of the minimisation of the number of animals used, refinement describes the minimisation of suffering; and replacement is defined by the avoidance of the use of living animals. Finally, animal-to-animal and experiment-to-experiment variability is reduced. Indeed, nasal explants from one pig can be infected in culture with different virus strains and samples can be taken at different time points post-inoculation. This allows comparison of viruses and study of the kinetics of primary virus replication within the same nasal mucosa.

In conclusion, porcine respiratory nasal explants can be maintained in culture at an air–liquid interface for at least 60 h with no significant morphometric changes or loss in viability. This creates an in vitro model that effectively resembles the in vivo situation. Susceptibility to two important porcine respiratory viruses, SHV1 (89V87) and swine influenza virus H1N1 (A/Sw/Belgium/1/98), was demonstrated.

Acknowledgements

This research was supported by a Concerted Research Action of the Research Council of Ghent University. We thank L. De Bels for her excellent preparation of all specimens for light and transmission electron microscopy, B. De Pauw for his excellent preparation of the specimens for scanning electron microscopy and M. Claeys for her help with handling the transmission electron microscope. David Lefebvre, Nick De Regge, Geert Opsomer and Fernand De Backer are acknowledged for their help with handling and euthanising the piglets.

References

Ali, M., Maniscalco, J., Baraniuk, J.N., 1996. Spontaneous release of submucosal gland serous and mucous cell macromolecules from human nasal explants in vitro. Am. J. Physiol. 270, L595–L600.

Appel, M.J., Menegus, M., Parsonson, I.M., Carmichael, L.E., 1969. Pathogenesis of canine herpesvirus in specific-pathogen-free dogs: 5–12-week-old pups. Am. J. Vet. Res. 30, 2067–2073.

Bradbury, P., Gordon, K., 1977a. Connective tissues and stains. In: Bancroft, J.D., Stevens, A. (Eds.), Theory and Practice of Histological Techniques. Churchill Livingstone, New York, pp. 109–110.

Bradbury, P., Gordon, K., 1977b. Connective tissues and stains. In: Bancroft, J.D., Stevens, A. (Eds.), Theory and Practice of Histological Techniques. Churchill Livingstone, New York, p. 103.

Fanucchi, M.V., Harkema, J.R., Plopper, C.G., Hotchkiss, J.A., 1999. In vitro culture of microdissected rat nasal airway tissues. Am. J. Respir. Cell Mol. Biol. 20, 1274–1285.

Gibson, J.S., Slater, J.D., Awan, A.R., Field, H.J., 1992. Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. Arch. Virol. 123, 351–366.

Gruenert, D.C., Basbaum, C.B., Widdicombe, J.H., 1990. Long-term culture of normal and cystic fibrosis epithelial cells grown under serum-free conditions. In Vitro Cell. Dev. Biol. 26, 408–411.

Jackson, A.D., Rayner, C.F., Dewar, A., Cole, P.J., Wilson, R., 1996. A human respiratory-tissue organ culture incorporating an air interface. Am. J. Respir. Crit. Care Med. 153, 1130–1135.

Jang, Y.J., Lee, S.H., Kwon, H.-J., Chung, Y.-S., Lee, B.-J., 2005. Development of rhinovirus study model using organ culture of turbinate mucosa. J. Virol. Methods 125, 41–47.

Johnson, L.G., Dickman, K.G., Moore, K.L., Mandel, L.J., Boucher, R.C., 1993. Enhanced Na+ transport in an air–liquid interface culture system. Am. J. Physiol. 264, L560–L565.

Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27, 137A.

Lin, C., Holland Jr., R.E., Williams, N.M., Chambers, T.M., 2001. Cultures of equine respiratory epithelial cells and organ explants as tools for the study of equine influenza virus infection. Arch. Virol. 146, 2239–2247.

Lopez-Souza, N., Avila, P.C., Widdicombe, J.H., 2003. Polarized cultures of human airway epithelium from nasal scrapings and bronchial brushings. In Vitro Cell. Dev. Biol. Anim. 39, 266–269.

Maeda, K., Horimoto, T., Mikami, T., 1998. Properties and functions of feline herpesvirus type 1 glycoproteins. J. Vet. Med. Sci. 60, 881–888.

Martineau-Doize, B., Caya, I., 1996. Ultrastructural characterization of the nasal respiratory epithelium in the piglet. Anat. Rec. 246, 169–175.

Nauwynck, H.J., Pensaert, M.B., 1992. Abortion induced by cell-associated pseudorabies virus in vaccinated sows. Am. J. Vet. Res. 53, 489–493.

Nauwynck, H.J., Pensaert, M.B., 1995. Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. Arch. Virol. 140, 1137–1146.

Nauwynck, H.J., 1997. Functional aspects of Aujeszky's disease (pseudorabies) viral proteins with relation to invasion, virulence and immunogenicity. Vet. Microbiol. 55, 3–11.

Pol, J.M.A., 1990. Interferons affect the morphogenesis and virulence of pseudorabies virus. Ph.D. Thesis. Utrecht, The Netherlands.

Russell, W.M.S., Burch, R.L., 1959. The principles of humane experimental technique. Methuen, Londen, UK.

Schierhorn, K., Brunnee, T., Paus, R., Schultz, K.D., Niehus, J., Agha-Mir-Salim, P., Kunkel, G., 1995. Gelatin sponge-supported histoculture of human nasal mucosa. In Vitro Cell. Dev. Biol. Anim. 31, 215–220.

Schmidt, D., Hubsch, U., Wurzer, H., Heppt, W., Aufderheide, M., 1996. Development of an in vitro human nasal epithelial (HNE) cell model. Toxicol. Lett. 88, 75–79.

Van Reeth, K., Brown, I., Essen, S., Pensaert, M., 2004. Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs. Virus Res. 103, 115–124.

Van Reeth, K., Nauwynck, H., 2000. Proinflammatory cytokines and viral respiratory disease in pigs. Vet. Res. 31, 187–213.

Whitcutt, M.J., Adler, K.B., Wu, R., 1988. A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. In Vitro Cell. Dev. Biol. 24, 420–428.

Wittmann, G., Jakubik, J., Ahl, R., 1980. Multiplication and distribution of Aujeszky's disease (pseudorabies) virus in vaccinated and nonvaccinated pigs after intranasal infection. Arch. Virol. 66, 227–240.

Wittmann, G., Ohlinger, V., Hohn, U., 1982. Vermehrung von Aujeszkyvirus (AV) in vakzinierten schweinen nach experimenteller infektion mit hohen und niederer virusmengen. Zentralbl. Veterinarmed. B 29, 24–30.

Wyler, R., Engels, M., Schwyzer, M., 1989. Infectious bovine rhinotracheitis/vulvovaginitis (BHV1). In: Wittman, G. (Ed.), Herpesvirus Diseases of Cattle, Horses, and Pigs. Kluwer Academic Publishers, Massachusetts, pp. 1–72.

Yoon, J.-H., Moon, H.-J., Seong, J.-K., Kim, C.-H., Lee, J.-J., Choi, J.Y., Song, M.S., Kim, S.-H., 2002. Mucociliary differentiation according to time in human nasal epithelial cell culture. Differentiation 70, 77–83.