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Differential Sensitivity of Well-Differentiated Avian Respiratory Epithelial Cells to Infection by Different Strains of Infectious Bronchitis Virus▽

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Infectious bronchitis virus (IBV) is an avian coronavirus affecting the respiratory tract of chickens. To analyze IBV infection of the lower respiratory tract, we applied a technique that uses precision-cut lung slices (PCLSs). This method allows infection of bronchial cells within their natural tissue composition under *in vitro* conditions. We demonstrate that IBV strains 4/91, Italy02, and QX infect ciliated and mucus-producing cells of the bronchial epithelium, whereas cells of the parabronchial tissue are resistant to infection. This is the first study, using PCLSs of chicken origin, to analyze virus infection. PCLSs should also be a valuable tool for investigation of other respiratory pathogens, such as avian influenza viruses.

Infectious bronchitis virus (IBV) is an avian coronavirus that affects the respiratory tract and that from there may spread to other organs, such as the kidneys and the reproductive system (3). It causes enormous economic problems in poultry flocks that are difficult to control by vaccines because of the occurrence of many different genotypes. Strains Italy02, 4/91, and QX have spread in recent years to different parts of the world (8). All three strains use alpha2,3-linked sialic acid as a receptor determinant (1, 7). In this respect, IBV resembles avian influenza viruses, most of which also have a preference for binding to alpha2,3-linked sialic acid. In a previous study we analyzed infection of the tracheal epithelium by the three IBV strains using tracheal organ cultures (TOCs). TOCs can be prepared by manual cutting of the trachea. A comparable culture system has not been described for the lower respiratory tract of avian species. Therefore, we adapted a technique that uses precision-cut lung slices (PCLSs), which has been described for some mammalian species, to the avian lung. PCLSs

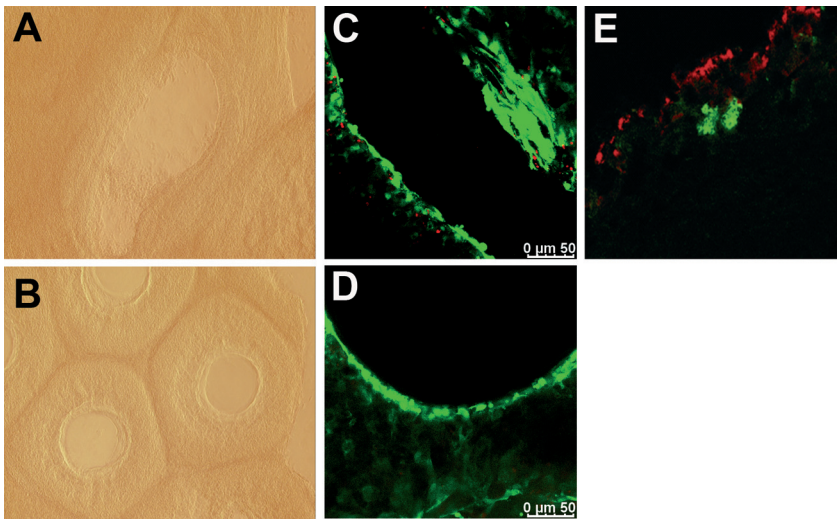

FIG. 1. Characterization of PCLSs. (A and B) Light microscopic pictures (phase contrast) of a bronchus (A) and parabronchi (B). Live and dead staining revealed that the majority of the bronchial epithelial cells are alive (green staining) directly after (C) and 1 week after (D) preparation. (E) Ciliated and mucus-producing cells were visualized using antibodies against ß-tubulin (red) and MUC5AC (green).

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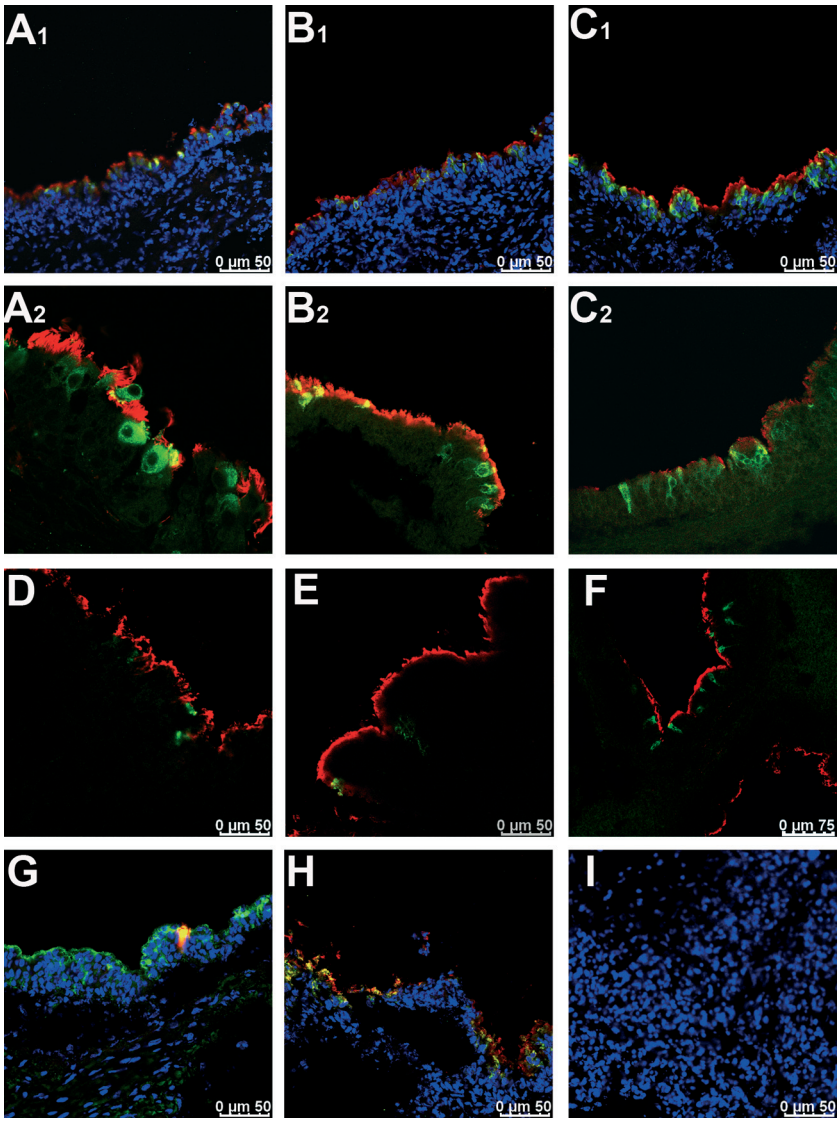

FIG. 2. Infection of bronchial epithelium by different IBV strains. Immunostaining for the presence of viral antigen (green) shows cells infected by strain 4/91 (A_1 and A_2), Italy02 (B_1 and B_2), or QX (C_1 and C_2). (A_1, B1, and C_1) Nuclei were stained with 4′,6-diamidino-2-phenylindole; (A_2, B_2, and C_2) nuclear staining was omitted. Ciliated cells were visualized by staining of ß-tubulin (red). PCLSs infected at a lower concentration (10^4 PFU/ml) are shown in panels D (4/91), E (Italy02), and F (QX). When 10^6 PFU/ml was applied, the bronchial epithelium of QX-infected PCLSs is already damaged (H). Goblet cells are sensitive target cells (G), as demonstrated by staining of a goblet cell with an anti-MUC5AC antibody (red) and an anti-IBV N protein antibody (green). Cells of the parabronchiolar tissue are not sensitive to IBV infection (I).

have recently been shown to be a valuable tool to study infections by respiratory viruses in the bovine system (5). The advantage of PCLSs is that they preserve the epithelial cells in their natural arrangement.

To adapt the PCLS technique to the chicken lung, we chose specific-pathogen-free (SPF) embryonated eggs as the organ source, because they are easier to handle than live SPF birds and are ideal in size; the whole lung fits into the slicing machine. Shortly before hatching, when the beak has entered the air cell, the embryos were killed by decapitation. A cannula was carefully inserted into the trachea, and the lungs were filled with low-melting-point agarose to stabilize the lung tissue. Freshly prepared lungs were cut with a Krumdieck tissue slicer into approximately 200-μm-thick slices. The slices were carefully washed to remove the agarose and were kept in an incubator at 37°C in eDulb medium (Gibco/Invitrogen). The slices comprise both bronchial tissue (Fig. 1A) and parabronchial tissue (Fig. 1B). The viability of the cells was monitored as described by Goris et al. (5). The criteria for viability were (i) ciliary activity observed under a light microscope, (ii) bronchoconstriction induced by addition of methacholine, and (iii) a live and dead staining of PCLSs showing that the majority of the epithelial cells lining the bronchus are alive (Fig. 1C and D). These features persist for about 1 week. Immunostaining revealed the presence of both ciliated cells (Fig. 1E, red) and mucus-producing cells (Fig. 1E, green).

To analyze the cells within a PCLS for sensitivity to infection

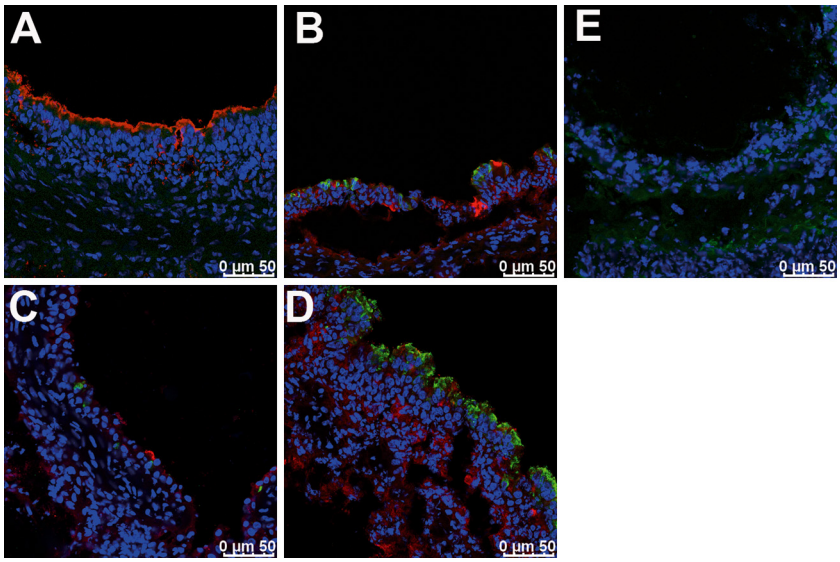

FIG. 3. Sialic acid expression in PCLSs. Staining with the lectin MAA is shown in red. Infected cells are shown in green; PCLSs were uninfected (A) or infected with strain 4/91 (B), strain Italy02 (C), or strain QX (D). No binding of the lectin SNA was detected In the bronchial epithelium (E).

by the different IBV strains, four PCLSs each in a well of a 24-well plastic plate were infected by either of the strains by applying an inoculum of 0.5 ml (10^5 PFU/ml) in eDulb medium. Four slices were analyzed for each strain, and all experiments were performed three times. Other concentrations of virus were also applied, but for comparison of the strains, we chose 10^5 PFU/ml because with a smaller amount of virus it was difficult to detect infection by strains 4/91 and Italy02 (Fig. 2D to F) and at larger amounts of virus the epithelium infected by strain QX was rapidly destroyed (Fig. 2H). After incubation for 8 h at 37°C, the slices were frozen in liquid nitrogen. To analyze the samples for the presence of viral antigen or cellular markers, cryosections were prepared from the PCLSs and stained with antibodies to visualize antigen via immunofluorescence. To detect IBV antigen, a monoclonal antibody, Ch/IBV 48.4, directed against the N protein was used. Infection was found to be restricted to the bronchoepithelial cells (Fig. 2A to C). Both cell types, ciliated cells (Fig. 2A to C) and mucus-producing cells (Fig. 2G), were sensitive to infection. This finding is in agreement with results obtained for the trachea (1, 7). No infected cells were detected within the epithelium lining the parabronchi (Fig. 2I). From this result we conclude that epithelial cells of the parabronchi are resistant to infection by IBV. Comparing the different strains, we found that the QX strain had a higher affinity for the bronchial epithelial cells than strains Italy02 and 4/91; i.e., under comparable conditions QX infected more cells (Fig. 2C and 3D) than the two other strains (Fig. 2A and B and 3B and C). This was obvious when we analyzed the cryosections at 8 h postinfection (Fig. 2A to C). If the inoculum was diluted 10-fold, infected cells were readily detected only with QX (Fig. 2F) but not with the other two strains (Fig. 2D and E). Similar results had been obtained with IBV-infected TOCs (1). Therefore, we conclude that strain QX is more efficient than strains 4/91 and Italy02 in infecting epithelial cells of the upper and lower airways. In a recent report, the pathogenicity of QX-like strains has been compared to that of strain 4/91 (2). The former strains were found to grow to higher titers and to cause more severe lesions (2). Therefore, susceptibility of epithelial cells in TOCs and PCLSs may be an indicator of the pathogenic potential of IBV strains.

Staining of PCLSs with the *Maackia amurensis* lectin II (MAAII) indicated that alpha2,3-linked sialic acid, a receptor determinant for IBV (5, 6), is abundantly expressed on the surface of the ciliated epithelium. Staining with the *Sambucus nigra* lectin (SNA) revealed that hardly any alpha2,6-linked sialic acids are expressed on the bronchial epithelium (Fig. 3E). Interestingly, we observed both in TOCs (1) and in PCLSs that the staining of the apical cell surface with MAAII was clearly reduced after infection of the epithelial cells by any of the three IBV strains (Fig. 3A to D). This indicates that the amount of alpha2,3-linked sialic acid expressed on the cell surface is reduced after infection. One may speculate that the sialic acids used as receptor determinants by IBV are internalized after binding of the viral particles. For IBV it has been shown that the virus enters the cells by a process that is dependent on low pH (4), suggesting receptor-dependent endocytosis as the entry strategy for IBV. The molecular basis and the relevance of the downregulation of surface-expressed sialic acids in the infected respiratory epithelium remain to be analyzed in the future. PCLS should also be a valuable tool to analyze infection by other avian respiratory viruses, such as avian influenza viruses and avian metapneumovirus.

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