The Journal of Microbiology, August 2005, p.366-369 Vol. 43, No. 4

NOTE

Cells in the Respiratory and Intestinal Tracts of Chickens Have Different Proportions of both Human and Avian Influenza Virus Receptors

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(Received May 17, 2005 / Accepted July 27, 2005)

Avian influenza viruses play a crucial role in the creation of human pandemic viruses. In this study, we have demonstrated that both human and avian influenza receptors exist in cells in the respiratory and intestinal tracts of chickens. We have also determined that primarily cultured chicken lung cells can support the replication of both avian and human influenza viruses.

Key words: influenza virus, receptor, chicken cells

All known hemagglutinin (HA) and neuraminidase (NA) subtypes of influenza A viruses are also known to circulate in populations of wild aquatic birds (Webster *et al*., 1992). Avian influenza viruses are occasionally transmitted to other hosts, including pigs, horses, humans, and domestic poultry. Avian influenza viruses, including H5N1, H9N2 and H3N2 subtypes, have been established in domestic poultry, and are known to be responsible for disease in both poultry and humans (Suarez *et al*., 1998; Campitelli *et al*., 2002; Li *et al*., 2003). Avian influenza viruses have also been shown to contribute to major influenza viral outbreaks in humans. The viruses associated with the 1918 pandemic originated from whole avian influenza viruses without reassortment, whereas the influenza viruses implicated in the 1957 and 1968 pandemics were generated as the result of reassortment occurring between avian and human influenza viruses in pigs (Scholtissek *et al*., 1978; Schafer *et al*., 1993; Reid *et al*., 1999, 2003).

Influenza viruses infect the cells via the binding of HA to the terminal sialic acids on the cell surface (Herrler *et al*., 1995). Human epithelial cells within the respiratory tract possess SAα2,6Gal-terminated sialylglycoconjugates (Baum *et al*., 1990), whereas the intestines of ducks possess SAα2,3Gal-terminated sialylglycoconjugates (Ito *et al*., 1998). In 1997, highly pathogenic H5N1 influenza viruses were transmitted directly from chickens to 18 humans, resulting in six deaths (Claas *et al*., 1998; Subbarao *et al*., 1998; Xu *et al*., 1999; Seo *et al*., 2002). Beginning in late 2003, outbreaks of highly pathogenic H5N1 viruses were reported in a host of Asian countries, including the Republic of Korea, Vietnam, Thailand, and China (Tran *et al*., 2004). In Thailand and Vietnam, 34 humans were infected by chickens infected with H5N1 influenza viruses, and 24 of these infected humans ultimately died. In 1999, avian H9N2 influenza virus subtypes, which are widespread in poultry in Asia (Lin *et al*., 2000), were transmitted from poultry to two children. These infected children suffered influenza-like symptoms, including fever, malaise, sore throat, headache, and vomiting (Peiris *et al*., 1999). The transmission of the H5N1 and H9N2 influenza viruses to humans suggests that chickens probably function as an alternative intermediate for the creation of pandemic influenza viruses. Until now, however, no reports have focused on the differing proportions of both avian and human influenza receptors in the respiratory and intestinal tracts of chicken. In this study, then, we attempted to determine what proportion of avian or human receptors are present in the respiratory and intestinal tracts of chickens, and whether the human influenza virus is capable of replication in chicken lung cells.

In order to determine the proportion of cells which express avian (SAα2,3Gal) or human (SAα2,6Gal) influenza receptors, we employed flow cytometric analysis, and assessed the proportion of cells expressing these influenza receptors (Fig. 1). This analysis was conducted using the Digoxigenin (DIG) Glycan Differentiation Kit (Roche, USA), with some modifications. Lung and colon tissues were trypsinized and resuspended at a concentration of 10^6 cells per ml in binding medium (Tris-buffered

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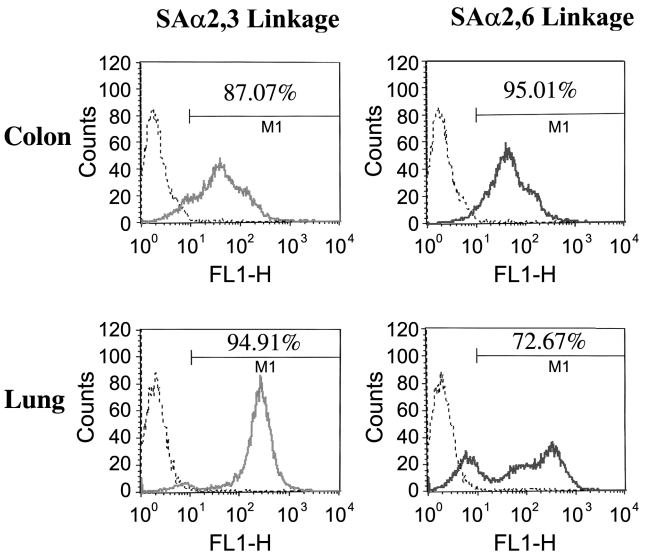

Fig. 1. Flow cytometric analysis of receptor specificity. Cells isolated from colons and lungs were stained with DIG-labeled lectins and fluorescein-conjugated anti-DIG antibodies. Dotted line, control stained only with fluorescein-conjugated anti-DIG antibodies.

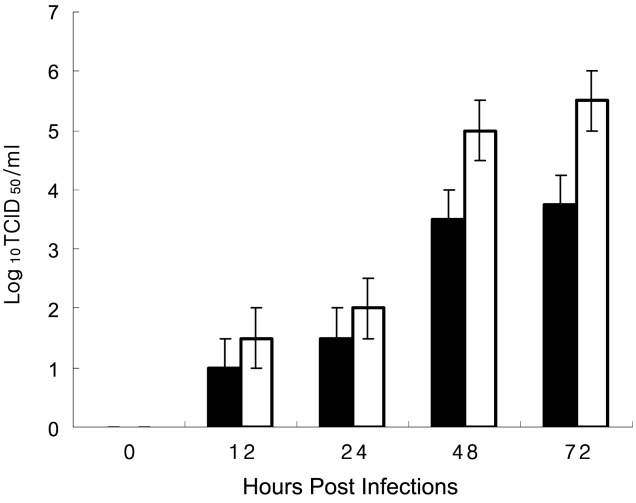

Fig. 2. Influenza virus replication in chicken lung cells. Primary cultured chicken lung cells were infected with the human influenza virus, A/Wyoming/3/2003 (H3N2), and the avian influenza virus, A/Chicken/Korea/S1/2003 (H9N2). The viral titers were determined by $\log_{10}$ $TCID_{50}$/ml. Filled square, A/Wyoming/3/2003 (H3N2); Open square, A/Chicken/Korea/S1/2003 (H9N2).

saline, pH 7.6, 0.5% BSA, 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 1 mM Mn^{2+}). The cells were incubated for 30 min at room temperature with DIG-labeled lectin, MAA (5 μg/ml), which binds specifically to SAα2,3Gal, and SNA (1 μg/ml), which binds specifically to SAα2,6Gal. The cells were then washed three times with cold PBS (pH 7.2) containing 1% horse serum and 0.05% Tween 20. FITC-labeled anti-DIG antibody diluted in PBS (pH 7.2) containing 1% horse serum was then added to the cells. After 30 min on ice, the cells were washed an additional three times with cold PBS (pH 7.2), supplemented with 0.05% Tween 20. The fluorescence intensity of the cells was analyzed using a FACS Calibur Fluorospectrometer (Becton-Dickinson, USA). The proportion of lung cells expressing human influenza receptors was determined to be lower than the proportion of lung cells expressing avian influenza receptors, whereas the proportion of colon cells expressing human influenza receptors was higher than the proportion of colon cells expressing avian influenza receptors. 87.07% of the colon cells tested positive for avian influenza receptors, and 95.01% of the colon cells tested positive for human influenza receptors.

Because chicken cells contain receptors for both avian and human influenza viruses, we attempted to determine whether or not the human influenza virus was capable of replication in the primary cultured lung cells of chickens. The lung tissues of 4-week-old white leghorn chicken were trypsinized in order to harvest the cells. Approximately 1×10^6 /ml of trypsinzed cells were plated in wells of 6-well plates with DMEM supplemented with 10% FBS, and were then incubated for 4 days at 37°C. The lung cells at confluence were infected with 0.01 plaque-forming units (PFU) of a human virus, A/Wyming/3/2003 (H3N2) and an avian virus, A/Chicken/Korea/S1/2003 (H9N2), in the presence of trypsin (0.3 μg/ml). Aliquots of the supernatants were collected at 24, 48, and 72 h post-infection, in order to determine the viral titers. The presence of viruses in the supernatants was assessed in MDCK cells, using the tissue culture infectious dose 50 ($\log_{10}TCID_{50}$/ml). Both the avian and human influenza viruses were found to have replicated in the lung cells, but the avian influenza virus, A/Chicken/Korea/S1/2003 (H9N2), exhibited growth characteristics superior to those of the human influenza virus, A/Wyoming/3/2003 (H3N2) (Fig. 2). At 72 h post-infection, the titers of human and avian influenza viruses were 3.75 $\log_{10}TCID_{50}$/ml and 5.5 $\log_{10}TCID_{50}$/ml, respectively. The patterns of growth exhibited by both of the viruses in primary colon cells were very similar to the growth patterns of both viruses in lung cells (data not shown).

Our study illustrated that chickens possess both avian and human influenza virus receptors in target cells in the lung and the colon. The receptor specificity in chickens, however, differs from that of ducks and horses. In the duck intestine, only the SAα2,3Gal linkages exist (Ito *et al.*, 1998). Avian influenza viruses replicate predominantly in the intestines of ducks, and large quantities of avian influenza viruses are known to be secreted via the feces of ducks. Additionally, influenza viruses with a receptor specificity for SAα2,3Gal linkage have recently been detected in horses. Both biochemical and immunohistochemical analyses and lectin-binding assays have demonstrated that epithelial cells in the tracheas of horses possess abundant SAα2,3Gal lectins (Suzuki *et al.*, 2000;

Lee *et al*, 2003). The dual receptor specificity observed in chicken cells suggests that chickens might function as an intermediate for the creation of influenza viruses with pandemic potential.

The proportion of influenza virus receptors in chicken colons is different from the proportion of receptors in the lungs. The chicken colon cells exhibit a higher proportion of α2,6Gal-linkage sialic acids than α2,3Gal-linkage sialic acids, whereas the lung cells exhibit a higher proportion of α2,3Gal-linkage sialic acids. In infected chickens, abundant quantities of avian influenza viruses are secreted in the feces. These results may help to explain how wholly avian influenza viruses, such as H5N1 and H9N2, can be transmitted directly from chickens to humans. Previous studies have demonstrated that target cells in chickens possess both avian and human influenza receptors, but none of these studies have attempted to determine the proportion of influenza virus receptors in the chicken cells (Gambaryan *et al*., 2002). H9N2 viruses recently isolated in China exhibit receptor specificity for α2,6Gal-linkage sialic acids (Matrosovich *et al*., 2001). The interspecies transmission of influenza viruses has been previously associated with the receptor specificity of the viruses (Rogers *et al*., 1983; Vines *et al*., 1998). Ducks have been reported to possess only the SAα2,3Gal linkage avian influenza virus receptor in their colons, where avian influenza viruses replicate predominantly (Ito *et al*., 1998; Ryu and Lee, 2004). In chickens, avian influenza viruses are known to replicate in both respiratory and intestinal tract cells. However, it remains to be determined how some avian influenza viruses, such as avian H9N2, actually obtain receptor specificity for α2,6Gal-linkage sialic acids. We hypothesize that some avian viruses may acquire this access by binding to α2,6Gal-linkage sialic acids in the colons or lungs of chickens. Once some avian viruses have succeeded in binding to α2,6Gal-linkage sialic acids, a population of viruses may evolve to possess single receptor specificity for α2,6Gal-linkage sialic acids, or for both α2,6Gal-linkage and α,2,3Gal-linkage sialic acids. Our results indicate that the continuous monitoring of avian influenza viruses for HA with receptor specificity for α2,6Gal-linkage sialic acids must be performed, in order to predict the next pandemic strains to emerge.

This work was supported by the internal initiative fund of Chungnam National University. We would like to express our appreciation and gratitude to Hyun Young Choi, for providing excellent technical support.

References

Baum, L.G. and J.C. Paulson. 1990. Sialyloligosacchrides of the respiratory epithelium in the selection of human influenza virus receptor specificity. *Acta Histochem. Suppl. Band* XL, 35-38.

Campitelli, L., C. Fabiani, S. Puzelli, A. Fioretti, F. Foni, A. De Marco, S. Krauss, R.G. Webster, and I. Donatelli. 2002. H3N2 influenza viruses from domestic chickens in Italy: an increasing role for chickens in the ecology of influenza? *J. Gen. Virol.* 83, 413-420.

Claas, E.C., A.D. Osterhaus, R. van Beek, J.C. De Jong, G.F. Rimmelzwaan, D.A. Senne, S. Krauss, K.F Shortridge, and R.G. Webster. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* 351, 472-477.

Gambaryan, A., R. Webster, and M. Matrosowitch. 2002. Differences between influenza virus receptors on target cells of duck and chicken. *Arch. Virol.* 147, 1197-1208.

Herrler, G., J. Hausmann, and H.D. Klenk. 1995. Sialic acid as receptor determinant of ortho-and paramyoviruses, p. 315-336. *In* A. Rosenberg (ed.), Biology of the Sialic Acids, Plenum Press, New York, NY.

Ito, T., J.N. Couceiro, S. Kelm, L.G. Baum, S. Krauss, M.R. Castrucci, I. Donatelli, H. Kida, J.C. Paulson, R.G. Webster, and Y. Kawaoka. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J. Virol.* 72, 7367-7373.

Lee, D., Y. Yoon, and C. Lee. 2003. Phylogenic analysis of the HIV-1 nef gene from Korean isolates. *J. Microbiol.* 41, 232-238.

Li, K.S., K.M. Xu, J.S. Peiris, L.L. Poon, K.Z. Yu, K.Y. Yuen, K.F. Shortridge, R.G. Webster, and Y. Guan. 2003. Characterization of H9 subtype influenza viruses from the ducks of southern China: A candidate for the next influenza pandemic in humans? *J. Virol.* 77, 6988-6994.

Lin, Y.P. and M. Shaw. 2000. Avian-to-human transmission of H9N2 subtype influenza A viruses: Relationship between H9N2 and H5N1 human isolates. *Proc. Natl. Acad. Sci. USA* 97, 9654-9668.

Matrosovich, M.N. 2001. H9N2 Influenza A viruses from poultry in asia have human virus-like receptor specificity. *Virology* 281, 156-162.

Peiris, M. and K.Y. Yuen. 1999. Human infection with influenza H9N2. *Lancet* 354, 916-917.

Reid, A.H., T.G. Fanning, J.V. Hultin, and J.K. Taubenberger. 1999. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. *Proc. Natl. Acad. Sci. USA* 96, 1651-1656.

Reid, A.H., T.A. Janczewski, R.M. Lourens, A.J. Elliot, R.S. Daniels, C.L. Berry, J.S. Oxford, and J.K. Taubenberger. 2003. 1918 influenza pandemic caused by highly conserved viruses with tworeceptor-binding variants. *Emerg. Infect. Dis.* 9, 1249-1253.

Rogers, G.N. and J.C. Paulson. 1983. Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* 127, 361-373.

Ryu, K. and S. Lee. 2004. Comparative analysis of interacellular trans-splicing ribozyme activity against hepatitis C virus internal ribosome entry site. *J. Microbiol.* 42, 361-364.

Schafer, J.R., Y. Kawaoka, W.J. Bean, J. Suss, D. Senne, and R.G. Webster. 1993. Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. *J. Virol.* 194, 781-788.

Scholtissek, C., W. Rohde, V. Von Hoyningen, and R. Rott. 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* 87, 13-20.

Seo, S.H., E. Hoffmann, and R.G. Webster. 2002. Lethal H5N1

influenza viruses escape host anti-viral cytokine responses. *Nat. Med.* 8, 950-954.

Suarez, D.L., M.L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D.E. Swayne. 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. *J. Virol.* 72, 6678-6688.

Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox. 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 279, 393-396.

Suzuki, Y. and T. Ito. 2000. Sialic acid species as a determinant of the host range of influenza A viruses. *J. Virol.* 74, 11825-11831.

Tran, T.H. and T.L. Nguyen. 2004. Avian influenza A (H5N1) in 10 patients in Vietnam. *N. Engl. J. Med.* 350, 1179-1188.

Vines, A. and K. Wells. 1998. The role of influenza a virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. *J. Virol.* 72, 7626-7631.

Webster, R.G., W.J. Bean, O.T. Gorman, T.M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* 56, 152-179.

Xu, X., K. Subbarao, N.J. Cox, and Y. Guo. 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: Similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 261, 15-19.