Influenza-B-Virus-Induced Eye and Brain Malformations during Early Chick Embryogenesis and Localization of the Viral RNA in Specific Areas

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Abstract
Influenza is prevalent worldwide, and the teratogenic effects of influenza infection have been suspected to occur within the developing central nervous system. We herein report the sequelae of influenza B viral infection during early chick embryogenesis. Chick embryos at Hamburger-Hamilton stage 9 were infected by an in ovo injection under the blastoderm of influenza B virus (B/Taiwan/25/99). At 48 h after infection, gross malformations of the eye and brain, ranging from 25 to 58% of 168 infected embryos, were observed, in contrast to 3-6% among 71 mock-infected controls (p < 0.0001 for both eye and brain malformations). Histological analyses showed extensive tissue degeneration and aggregates of cells in the head mesenchyme, suggesting cell death and heterotopia. Influenza B viral RNA was directly localized by in situ hybridization with probes specific for the HA segment. Viral RNA was extensively detected in the head surface ectoderm and in the lung bud. In the developing brain, viral RNA was specifically located in the anterior neural retina, habenular area, mid-thalamus, and rhombencephalon. Our data show that influenza B virus can be a teratogenic agent in neural and nonneural embryonic tissues, raising concern for transplacental infection during early pregnancy.

Introduction
Influenza viruses remain one of the most significant and prevalent pathogens worldwide, and many efforts have been made in describing the molecular aspects of these viruses, in elucidating their epidemiology and modes of spread, and in developing strategies for prevention and treatment. Nevertheless, whether maternal influenza viral infection leads to teratogenesis in the fetus remains controversial, and how the virus affects normal embryogenesis is largely unknown.

Prenatal exposure to influenza epidemics has been reported by epidemiological studies to increase the risk of adult schizophrenia in Finland [23], England and Wales [29], and Japan [18]. However, a study in the US did not find this to be true [41]. Another study in the US [21] and a Scottish study showed no difference in levels of schizophrenia between subjects exposed and those not exposed to influenza [17]. These contradictions likely result from methodological variations in gathering information about maternal infection based on maternal memory or the...
quality of later psychiatric diagnoses [44]. In addition, excessive drug consumption contingent on infection may have complicated the acceptance of a viral disease as being teratogenic in an epidemiological study [16]. Eventually, influenza-caused neuropsychiatric defects have to be characterized by studying the underlying mechanisms responsible for the abnormalities during neurogenesis.

Many efforts have been made to elucidate maternal postinfluenza encephalitis in relation to fetal neurological defects, based on serological detection of maternal antibodies or, more directly, the spread of viral antigens in the maternal serum [7–9, 31, 40]. The rationale is that the presence of maternal antibodies against influenza virus and a detected level of viral antigens in the maternal circulation would indicate transplacental effects of influenza virus on the fetus. To our knowledge, however, none of the previous studies directly located the distribution of influenza viral RNA. Thus, the effect of direct access, replication, and targeting of the influenza viral genome in early developing embryos or fetuses has not been shown and whether nonneural embryonic tissues can be targeted has not been elucidated. In addition, previous studies concentrated on maternal exposure during the second trimester [1, 36], by which time the potential effects on early neurogenesis in the fetus would have passed.

With the difficulties in studying human subjects, attempts to correlate influenza viral infection with congenital neuropsychiatric disorders have to be shifted to animal models. To elucidate the causal effects, direct localization of the distribution of the viral genome would be more informative than would antigen or antibody detection. In the present study, we investigated the effect of influenza B infection on early chick embryogenesis and directly localized the distribution of the influenza B viral genome in the developing embryos by in situ hybridization.

**Materials and Methods**

**Virus Propagation and Titration**

The influenza virus B/Taiwan/25/99 strain was isolated from a child who exhibited neurological symptoms during an epidemic in Taiwan in 1999. The identity of the virus was previously confirmed by nucleic acid sequencing. The virus was obtained as a kind gift from Dr. Shin-Ru Shih, Chang Gung University, Taiwan, ROC. The virus was grown at 37°C with 5% CO2 in Madin-Darby canine kidney (MDCK) cells in Eagle's modified essential medium (EMEM) (Gibco, Grand Island, N.Y., USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The virus titer was determined by plaque assay on MDCK cells. After titration, the influenza B virus was diluted in EMEM to a concentration of 5 × 10^8 plaque-forming units (PFU)/ml and stored in at −70°C. The virus preparations were thawed shortly before use, and no refrozen virus was used.

**Preparation and Infection of Chick Embryos**

Fertilized, pathogen-free chicken eggs were purchased from a local farm (Jin-Dan Incubation Services, Taichung County, Taiwan, ROC) and incubated at 38.5°C in order for the embryos to develop to Hamburger-Hamilton stage 9 [11]. To infect a chick embryo, an aliquot of 20 μl of the prepared influenza B virus was carefully injected in ovo into the sub-blastodermal space, i.e. between the embryo and its underlying yolk, in the area opaca outside of the more translucent central area pellucida (fig. 1). A 30-gauge needle was used for injection. Efforts were made to avoid any damage to the embryos, and during the procedure, there was essentially no direct contact between the injection needle and the embryo. Mock injections of 20 μl of EMEM without virus were used as controls. Infected and noninfected chick embryos were returned to separate incubators at 38.5°C for further incubation until 48 h after infection. In total, 168 infected chick embryos and 71 noninfected controls were used for observation of gross malformations and histological analyses. For in situ hybridization analyses, another 12 influenza-B-virus-infected embryos and 6 noninfected controls were collected at time intervals of 12, 24, 36 and 48 h following infection and mock injections.

**cDNA Cloning of an Influenza-B-Specific HA Segment**

To directly localize the presence and replication of influenza B viral RNA in embryos following infection, cDNA of the HA segment was cloned, and probes for both negative and positive RNA strands were synthesized for detection. The influenza B viral total RNA was...