Susceptibility of Immune-Suppressed Pigeons to Subtypes H5N2 and H6N1 Low Pathogenic Avian Influenza Virus

Tsung-Hsiu Fang, Yi-Yang Lien, Ming-Chu Cheng, and Hsiang-Jung Tsai

SUMMARY. Susceptibility to infection with avian influenza virus (AIV) was studied in healthy and immune-suppressed pigeons, which were treated with the immunosuppressant cyclophosphamide (CY) before infection. Two subtypes of low pathogenic AIV (LPAIV; CK/TW/H5 and CK/TW/H6) were inoculated via the oculonasal route. Nested reverse transcriptase-polymerase chain reaction (nested RT-PCR) and virus isolation were used as detection methods. The CY-treated and -untreated pigeons in both experiments did not shed viruses or become antibody positive throughout the 21-day observation period. All pigeons were negative for AIV RNA when trachea, lung, pancreas, spleen, kidney, and rectum tissues were examined. Negative results were also obtained in uninoculated contact chickens, which were housed together with H6N1 AIV-inoculated pigeons. Therefore, it was concluded that the pigeons are resistant to infection with these two LPAIVs and do not serve as transmission hosts, even in the presence of immune dysfunction.

Key words: avian influenza, pigeon, cyclophosphamide, nested RT-PCR, immunosuppression

Abbreviations: AIV = avian influenza virus; CY = cyclophosphamide; DPI = days postinoculation; EID_{50} = 50% embryo infective dose; HI = hemagglutinin inhibition; HPAI = highly pathogenic avian influenza; LPAI = low pathogenic avian influenza; NP = nucleoprotein; RT-PCR = reverse transcriptase-polymerase chain reaction; SPF = specific pathogen free

Avian influenza viruses (AIVs) are classified in the family Orthomyxoviridae, genus Influenzavirus A (15). The very virulent viruses cause fowl plague, now termed highly pathogenic avian influenza (HPAI), carrying a mortality that may be as high as 100%. These viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI. All other viruses that cause a much milder, primarily respiratory disease are designated low pathogenic avian influenza (LPAI) viruses. This mild disease may be exacerbated by other infections or environmental conditions (1). AIVs have been isolated from more than 90 species of free-living aquatic birds (15). The worldwide concern about the spread of AI among humans and poultry also has many pigeon fanciers interested in the role of pigeons, especially racing pigeons, whereas there are conflicting reports on the susceptibility of pigeons.

Pigeons intranasally inoculated with HPAIV (H5N1) lack gross and histologic lesions, viral antigen, and reisolation of virus (13). Intravenous and intranasal inoculation of pigeons with HPAIV (H5N9) resulted in seroconversion but not virus shedding or clinical disease, and noninoculated turkeys placed as contact controls remained clinically healthy and seronegative (9). In contrast, another study conducted with the same H5N9 virus resulted in the death of 1 of 19 intranasally inoculated pigeons and virus reisolation from the tracheas of two pigeons. But only one pigeon showed questionable seroconversion (14). Infection of pigeons with H7 viruses results in only some of them showing clinical signs, virus shedding, and seroconversion (6); however, in another study, pigeons inoculated with HPAIV (H5N2 and H7N7) remained apparently healthy throughout the 21-day observation period, did not shed viruses on 3, 7, 14, and 21 days postinoculation (DPI), and had no demonstrable levels of antibodies on 21 DPI (11).

In the year 2000, an epidemiological study on the health status of free-living pigeons in the city of Ljubljana, Slovenia, failed to detect antibodies to avian influenza virus in the serum of 139 pigeons (3). Nevertheless, surveillance in a live-poultry market in Nanchang, South Central China, during January 2000 through April 2001, found subtypes H3N3, H3N6, and H9N2 AIVs in 0.5% of pigeon samples (6/1190) (8). In 2000, one H6N1 strain AIV was isolated in Taiwan from an imported pigeon (Cheng, unpublished data). Furthermore, in 2004, pigeons were discovered infected with H5N1 HPAIV, designated as A/pigeon/Samut Prakan/Thailand/CU-202/04 (GenBank

Corresponding author.
accession numbers DQ083693, DQ083655, DQ083619, and DQ083583) in Thailand, and as a result of this outbreak, more than 400 pigeons had to be culled as a control measure (11).

In Taiwan, H6N1 AIV has been shown to associate with mortality in poultry (16). The H5N2 subtype of LPAIV was detected in Taiwan in 2004, and as a control measure, 383,852 birds were destroyed (10). In both cases, the possible role of the pigeons in the spread of AIV involved in the outbreaks of the poultry flocks was of great concern to the regulatory authority. Because of the conflicting observations on AIV infection and surveillance, we attempted to enhance virus infection by altering the immune status of pigeons using cyclophosphamide (Cy). Cy is an immunosuppressive drug commonly used in immunological experiments. Numerous authors have shown that Cy is primarily a B-cell suppressor, but it also produces transient T-cell deficiency (4,5). The purpose of this study was to determine whether immunosuppressed pigeons become infected when exposed to LPAIV involved in the outbreaks of the poultry flocks by the oculonasal route and are able to transmit the infection to contact pigeons or chickens.

**MATERIALS AND METHODS**

**Pigeons.** Clinically healthy male and female racing pigeons between 3 and 24 mo of age and free of antibodies to H5 and H6 AIV were used. Preinoculation oropharyngeal and cloacal swabs were collected from all pigeons to ensure that these birds were not harboring AIVs. They were maintained in cages under negative air pressure and continuous light. Throughout the experimental period, feed and water were provided ad libitum.

**Chickens.** Clinically healthy 7-wk-old chickens used for Expt. 2 were obtained from a specific-pathogen-free (SPF) flock (white Leghorn, National Research Institute for Animal Health, Council of Agriculture, Executive Yuan, Taiwan), and feed and water were provided ad libitum.

**Avian influenza viruses.** A/chicken/Taiwan/1209/03 (LPAIV H5N2) (CK/TW/H5) was used to inoculate the pigeons in Expt. 1, and A/chicken/Taiwan/3152/03 (LPAIV H6N1) (CK/TW/H6) was used to inoculate the pigeons and chickens in Expt. 2. CK/TW/H5 was isolated by Dr. M. C. Cheng (National Research Institute for Animal Health) from affected chickens involved in the outbreak of H5N2 LPAI that occurred in Changhua, Taiwan, during the end of 2003. CK/TW/H6 was provided by Dr. C. W. Wang (Department of Veterinary Medicine, National Taiwan University, Taiwan) and was isolated from broiler breeders in Taichung, Taiwan, in 2003. The 50% embryo infective doses (EID50) per milliliter of these two virus preparations were 5 × 10^3 EID50/ml.

**Cy treatment.** The dosage of Cy treatment reported by Coignoull and Vindevogel (2) was used in this study and was about 22 mg/kg/day for 4 days. The pigeons in Expts. 1 and 2 were given 10 mg of Cy (Endoxan, Baxter, Unterschleissheim, Germany) per day, intramuscularly, for 4 days. The chickens in Expt. 2 were injected intramuscularly with 50 mg of Cy for 4 days. Birds were treated before inoculation in both experiments.

**Experimental design.** In Expt. 1, 38 pigeons were randomly allocated into six groups: 1) Cy treated + virus (n = 10), 2) no Cy treated + virus (n = 10), 3) Cy treated + contact (n = 10), 4) no Cy treated + contact (n = 10), 5) Cy treated + no virus (treatment control; n = 10), and 6) no Cy treated + no virus (negative control; n = 10). Pigeons in groups 1 and 2 were inoculated individually via the oculonasal route with 0.1 ml of CK/TW/H5 virus, and pigeons in groups 3, 4, 5, and 6 were inoculated with 0.1 ml of saline via the same route.

In Expt. 2, 24 pigeons were randomly allocated into three groups: 1) Cy treated + virus, 2) no Cy treated + virus, and 3) Cy treated + virus + contact; and 21 chickens were divided into five groups: 1) no Cy treated + contact (n = 5), 2) Cy treated + virus (n = 5), 3) no Cy treated + virus (n = 5), 4) no Cy treated + no virus (negative control; n = 3), and 5) Cy treated + no virus (treatment control; n = 3). Pigeons in group 3 and chickens in group 1 were placed in the same room. Pigeons in groups 1, 2, and 3 and chickens in groups 2 and 3 were inoculated individually via the oculonasal route with 0.1 ml of CK/TW/H6 virus. Chickens in groups 4 and 5 were inoculated with 0.1 ml of saline via the same route.

At 3, 7, 14, and 21 DPI, all birds in each experiment were bled, and oropharyngeal and cloacal swabs were collected separately in 2 ml of brain heart infusion medium, then stored at −20°C. All birds were euthanized and necropsied at 21 DPI. Trachea, lung, pancreas, spleen, kidney, and rectum were taken and stored at −20°C. Birds that died during the observation period were also necropsied.

**RNA isolation.** RNA was extracted using Trizol reagent (Gibco BRL, Grand Island, NY). In brief, 0.1 ml of swab fluid or organ homogenate was mixed completely with 1 ml of Trizol reagent and the mixture kept at room temperature for 5 min. Exactly 200 μl of chloroform (Amresco, Solon, OH) was added and the sample was vortexed and incubated for an additional 3 min. The aqueous and organic phases were separated by centrifugation at 10,000 × g for 15 min, and the RNA was precipitated from the aqueous phases by adding an equal volume of isopropanol (Amresco). The RNA precipitate was collected by centrifugation at 10,000 × g for 20 min, washed by 75% ethanol, and dissolved in 30 μl of RNase-free water.

**Nested reverse transcription-polymerase chain reaction (RT-PCR).** Nested RT-PCR was carried out with the primer pair developed previously for the detection of the nucleoprotein (NP) gene of AIV (7). The first NP-specific primer pair was NP1039 (forward): 5'-TGGATGCGATTC(C/A)ATTCGC and NP1529 (reverse): 5'-GCAATGTCTCCGAAAGATAAG. RT-PCR assays were performed as described previously (7). The first RT-PCR product was subjected to a second (nested) PCR, which used primer pair NP1200f: 5'-CAAG(A/G)TAATGCGG(A/C/T/Q)ATAAG(A/G)AC and NP1490r: 5'-CTCATGTG(C/A)AGGAGGCAC (7). The nested RT-PCR assays were carried out with 1 μl of the first-round amplification product and 29 μl of reaction mixture, including 3 μl of 10-times reaction buffer (Viogene, Sunnyvale, CA), 1 μl of dNTP mix (10 mM each of four dNTPs, Viogene), 0.5 μl of Taq DNA polymerase (5 U/μl, Viogene), 0.3 μl of each primer (50 μM each), and 23.9 μl of water. The nested RT-PCR condition for the amplification of NP was 95°C for 3 min, 35 cycles of 95°C for 30 sec, 55°C for 40 sec, and 72°C for 40 sec, followed by 72°C for 10 min.

**Serology.** Serum samples were tested for the presence of H5 or H6 subtype AIV antibody by hemagglutinin inhibition (HI) test.

**Virus isolation.** Swab and tissue samples of pigeons in Expts. 1 and 2 were frozen and thawed three times and the extracted fluid were collected and clarified by low-speed centrifugation. The supernatant was filtered through a sterile membrane filter with a 0.45-μm pore size, and 0.2 ml of the filtrate was inoculated into the allantoic cavity of each of five 9- to 11-day-old embryonated SPF hens’ eggs. Inoculated eggs were incubated at 37°C for 96 hr. After that, allantoic fluids were harvested and tested for hemagglutination activity. When necessary, the allantoic fluids were passaged again once or twice in embryonated eggs.

**Histology.** To determine whether Cy could damage the immune system, thymus and bursa of Fabricius were collected from some of the Cy-treated and untreated pigeons at 3 and 21 DPI of Expt. 2, fixed in 10% buffered formalin. All tissue samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for microscopic examination.

**RESULTS**

**Expt. 1.** Though 2 of 10 pigeons in group 1 died on 6 and 9 DPI, none of the other pigeons inoculated via oculonasal route with H5N2 virus or saline or via contact with other pigeons showed signs of illness or died during the experimental 21-day period. All pigeons in the six groups (including the two dead pigeons) were negative for AIV RNA when oropharyngeal swabs, cloacal swabs, trachea, lung, pancreas, spleen, kidney, and rectum tissues were tested by nested RT-PCR, and no AIV was recovered in virus isolation from inoculated pigeons on 3, 7, 14, and 21 DPI, even after the third passage in embryonated eggs. Also, all pigeons were seronegative for antibodies to H5 subtype AIV (Table 1).
CK/TW/H5 virus. The HI titer ranged between 26 and 28, RT-PCR. The inoculated chickens in groups 2 and 3 also produced noninoculated chickens, which were placed as contact controls in 3, 7, 14, and 21 DPI. Negative results were also obtained in tissues from all pigeons by nested RT-PCR or by virus isolation on a dead pigeon, did not shed viruses or become antibody positive throughout the 21-day observation period. None of the AIVs could be detected in trachea, lung, pancreas, spleen, kidney, and rectum throughout the 21-day observation period. None of the AIVs could be detected in oropharyngeal and cloacal swabs and/or tissues. Moreover, none of the non-Cy-treated virus-inoculated pigeons showed clinical signs or died. It is postulated that the death of three birds might be simply due to the toxic effect of Cy or due to susceptibility of the pigeons to other unknown harmful factors under immunosuppressive conditions.

**Expt. 2.** One of 10 pigeons in group 3 died on 3 DPI, but neither morbidity nor mortality was observed in other pigeons in groups 1, 2, and 3. All pigeons in the three groups, including the dead pigeon, did not shed viruses or become antibody positive throughout the 21-day observation period. None of the AIVs could be detected in trachea, lung, pancreas, spleen, kidney, and rectum tissues from all pigeons by nested RT-PCR or by virus isolation on 3, 7, 14, and 21 DPI. Negative results were also obtained in noninoculated chickens, which were placed as contact controls in group 1, and in the chickens in groups 4 and 5. However, the results were different for chickens in groups 2 and 3; AIV could be detected in both oropharyngeal and cloacal swabs on 3 and 7 DPI by nested RT-PCR. The inoculated chickens in groups 2 and 3 also produced detectable levels of antibody. The HI titer ranged between 2^3 and 2^8, and there were no apparent titer differences between Cy-treated and nontreated groups (Table 1).

**Histology.** The thymus of Cy-treated pigeons on 3 DPI showed depletion of lymphocytes in cortex and medullary areas with the proportion of degenerating lymphocytes increasing. Nevertheless, microscopic evaluation of thymus from Cy-treated pigeons on 21 DPI revealed no morphologic alterations.

The bursa of Fabricius of Cy-treated pigeons was visibly smaller than that of nontreated pigeons on gross examination. Microscopically, in normal bursa, the connective tissue surrounding the follicles consisted of a fine network of collagen fibers, and the supporting satellite reticulo-epithelial cells of the follicles were not visible due to the intense lymphoid cell density. In the Cy-treated pigeons, the interfollicular connective tissue widened and the cortex and medulla showed moderate to severe lymphocytic depletion on 3 and 21 DPI. Marked atrophy and fibroplasia of the follicles were also revealed in Cy-treated pigeons.

Besides thymus and the bursa of Fabricius, no apparent morphologic lesions were observed both in AIV inoculated and non-inoculated pigeons.

**DISCUSSION**

The marked lymphocytic depletion in lymphoid tissues of Cy-treated pigeons in this study appears to be similar to that observed by other workers (2,4,5). Cy induced an overall cytotoxic effect on both B- and T-lymphoid cells, but the T-cell system regenerates more rapidly (2). In our experiment, lesions of thymus from Cy-treated pigeons on 3 DPI were characterized by lymphocyte degeneration and depopulation, but revealed no morphologic alterations on 21 DPI. However, Cy treatment in pigeons produced a more severe atrophy and a more intense depletion of lymphoid cells in bursa of Fabricius than in thymus on 3 and 21 DPI. The result is in accordance with previous observations that Cy principally destroys B cells and results in chemical bursectomy, and that the effect to T cell and thymus are transient (2,4,5).

Although two Cy-treated pigeons inoculated with H5N2 virus in Expt. 1 died on 6 and 9 DPI and one Cy-treated pigeon inoculated with H6N1 virus in Expt. 2 died on 3 DPI, none of the AIVs could be detected in their specimens by nested RT-PCR or virus isolation, and no seroconversion was detected by HI assays. Moreover, none of the non-Cy-treated virus-inoculated pigeons showed clinical signs or died. It is postulated that the death of three birds might be simply due to the toxic effect of Cy or due to susceptibility of the pigeons to other unknown harmful factors under immunosuppressive conditions.

In the present experiment, the surviving pigeons in both experiments remained clinically healthy and did not shed viruses or become antibody positive throughout the 21-day observation period. All pigeons were negative for AIV RNA when trachea, lung, pancreas, spleen, kidney, and rectum tissues were tested. Transmission of AIV to contact pigeons did not occur. The negative results were also obtained in noninoculated chickens that were used as contact controls in the H6N1 AIV experiment. The results showed that the Cy-treated and nontreated pigeons under the conditions of the study were not infected with CK/TW/H5 and CK/TW/H6 AIV when inoculated via the ocular nasal route. Therefore, it was concluded that the pigeons are resistant to infection with these two LPAIVs and do not serve as transmission hosts, even in the presence of immune dysfunction, which might be induced by stress situations such as infectious diseases, severe feeding restriction, poor nutrition, crowding, and intensive training, especially of racing pigeons. Despite these reassuring findings, because of the small number of birds and isolates used in this study, the roles of pigeons in the transmission of other AIVs, especially the H5N1 strains HPAIV endemic in several Asian countries since the end of 2003, still need to be determined. Also the facts that free-flying domestic pigeons could act as mechanical vectors and vehicles for long-
distance transmission of any influenza A virus if their plumage or feet were contaminated should be considered in the AIV control programs (6).

REFERENCES


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