

Prevention of Highly Pathogenic Avian Influenza A/H5N1 Infection by Passive Immunotherapy Using Antiserum

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Abstract

The rapid epidemic of highly pathogenic A/H5N1 avian influenza virus by transmission from poultry to humans triggered global unrest in the pandemic of novel influenza. If a human trophic strain of avian influenza viruses replicates in livestock including pigs and chickens, it may have high infectivity and pathogenicity to humans. The most effective method of reducing the outbreaks of influenza would be prophylaxis with an effective vaccine as well as anti-viral drugs including Oseltamivir and Zanamivir hydrate. In this study, chicken antiserum against A/H5N1 virus was produced: the antisera from immunized adult chicken had a strong binding activity to A/H5N1 viral antigens by ELISA. Furthermore, the antiserum strongly inhibited hemagglutination of erythrocytes and cytopathic effects in MDCK cells, indicating a strong neutralization activity against A/H5N1 infections. Interestingly, the mortality rate of chicks inoculated with A/H5N1 virus was dramatically decreased with the antiserum injection. These results suggest that antiserum may be a potentially effective protective and therapeutic modality for A/H5N1 infection.

Keywords

Avian Flu, Influenza Virus, H5N1, Antiserum, Chicken

1. Introduction

Avian influenza is most often spread by contact between infected and healthy birds, although it can also be spread indirectly through contaminated equipment [1] [2] [3]. Highly pathogenic strains of avian influenza viruses transmit imme-

diately among birds and can destroy a flock within one day. In recent years, cases of highly pathogenic avian influenza virus A/H5N1 infections in human have increased, mainly in Southeast Asia [4] [5]. However, at the moment, no pandemic strain of A/H5N1 virus has yet been found.

Vaccination is considered extremely effective for prevention of infectious diseases including influenza virus infections [6]. However, vaccination during a pandemic is thought to be inappropriate for suppressing infection because considerable time is needed to acquire immunity against antigens [7] [8]. A/H5N1 vaccines for domestic fowl have been developed and are sometimes used, although there are many difficulties associated with these vaccines and their administration, making it difficult to decide whether they help or hurt [9]. A/H5N1 human pandemic vaccines and technologies to rapidly create them are in the H5N1 clinical trial stage but cannot be verified as useful until a pandemic strain has been identified. Immunotherapy is performed based on passive or acquired immunity [10] [11] [12]. Passive immunotherapy involves administering antisera or antibodies prepared in advance to patients and has an immediate effect on preventing and treating infections. For example, snakebite victims can be saved by inoculating a neutralizing antiserum against snake venom [13]. Acquired immunotherapy is primarily achieved through vaccination. Each of these therapies has its advantages and disadvantages, but passive immunotherapy is superior when immediate efficacy is sought.

Chickens are a major source of protein for humans and one of the most common and widespread domestic animals. Young chickens are susceptible to several infectious diseases, including avian influenza, avian encephalitis, pullorum, Marek's disease, laryngotracheitis, and infectious bronchitis [14] [15]. In chickens, avian flu has out broken on a global scale, but in various avian flu-free countries, vaccinating domestic fowls is not allowed [16]. In such countries, the current method of preventing infection is to destroy infected animals as well as those suspected of being infected. In southeast Asia, millions of domestic birds have been slaughtered to prevent the spread of the virus. As such, an outbreak of avian influenza will deliver huge economic losses to poultry farmers [17] [18] [19]. Thus, the development of a novel method for preventing influenza aside from vaccination is desired.

In this study, in order to cope with a potentially highly pathogenic avian influenza pandemic, antiserum, which is a form of passive immunotherapy not involving vaccination, was administered to chickens, and the effect of suppressing infection by highly pathogenic avian influenza was examined.

2. Material & Methods

The summary of this study is shown in **Figure 1**. The antiserum was sampled from adult chicken after immunization with an inactivated vaccine strain of A/H5N1 antigen. After antibody titration in the antiserum was confirmed by ELISA, hemagglutination (HA) test and neutralization assays in culture cells, the antiserum was injected to infant chicks prior to high pathogenic avian influenza

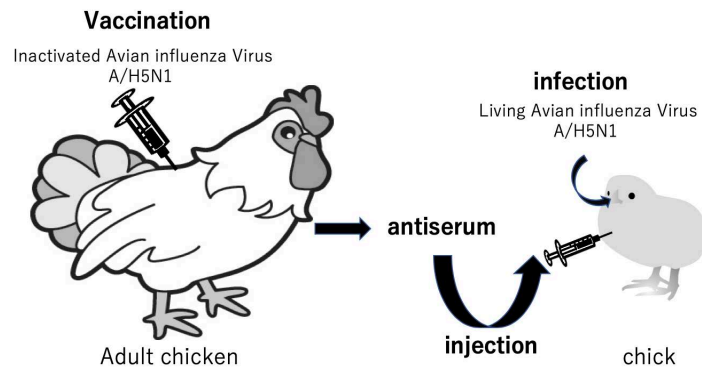


Figure 1. Schematic diagram of this experiment. Antiserum was produced in adult chickens by immunization with inactivated A/H5N1 virus. The antiserum was separated from the chicken blood and injected intramuscularly to chicks. The chicks were then inoculated with A/H5N1 virus, and the effects of antiserum administration on infections were verified.

virus A/H5N1 infection, and then the survival rate and histopathological findings of infected chickens were verified.

2.1. Generation of Antisera against A/H5N1 Virus

Adult male chickens (White Leghorns) with no vaccination history were used to produce antiserum against avian influenza virus. At our laboratory, an inactivated Indonesian vaccine strain of A/H5N1 antigen (50PD₅₀/bird) was mixed with an adjuvant and inoculated into the pectoral muscle of the chicken. Four weeks later, blood samples were collected from the chicken, and the serum was separated by centrifugation. Antibody titers of the obtained antiserum were measured by an enzyme-linked immunosorbent assay (ELISA). Furthermore, the neutralizing activity against avian influenza virus was scored by a hemagglutination inhibition (HI) test using erythrocytes [20].

2.2. ELISA

Based on our previous papers, binding ability of the chicken antisera to the viral antigen was measured by ELISA [20]. Each well of 96-well ELISA plates (Sumitomo Bakelite, Japan) was coated with antigens of pandemic influenza virus A/H1N1 (A/California/9/2009 (H1N1) pdm09) or avian A/H5N1 virus in phosphate-buffered saline (PBS), and the ELISA plate was stored overnight at 4°C. After washing the wells twice with PBS containing 0.05% Tween 20, each of the subsequent incubation steps were performed. A commercial blocking buffer (DS Pharma Biomedical, Japan) were added into the wells and incubated at 37°C for 2 h. Serial dilutions of preimmune or antiserum were added vertically to the wells and incubated at 37°C for 1 h. Then, horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit IgG against chicken IgY Fc fraction) (Nakarai Tesque, Japan) diluted in PBS (1:5000) was dispensed into each well, and incubated for 1 h at 37°C. Next, a substrate buffer containing TMB (Sumitomo Bakelite, Japan) was added to each well, and samples were incubated at 37°C for 15 min.

The color development reaction was stopped by the addition of a stopping reagent (1.25 M sulfuric acid). The absorbance was measured at 450 nm using the ELISA plate reader.

2.3. Hemagglutination (HA) Test

Whole blood from guinea pigs was washed with PBS, and the erythrocytes were prepared after centrifugation. Serial 2-fold dilutions of pandemic influenza virus A/H1N1 or A/H5N1 viruses were mixed with erythrocyte solutions in clear 96-well micro test polystyrene assay plates (Becton Dickinson, USA). After 45 minutes at room temperature, the hemaggregation activity was observed, and the HA titers of the virus were measured. The highest dilutions (indicating clear hemaggregation) were scored as HA titers [20].

2.4. HI Test

Serial dilutions of preimmune or antiserum were mixed with 8-HA units of the influenza viruses in clear 96-well micro test polystyrene assay plates (Becton Dickinson, USA). After 30-minute incubation at room temperature, erythrocytes were added, pipetted gently, and then incubated for another 45 minutes at room temperature. The hemaggregations in each well were observed, and the HI titers were scored based on the HA titer with preimmune serum/HA titer with antiserum (a higher ratio indicates a stronger inhibitory activity of the antiserum against the pandemic influenza virus) [20].

2.5. Neutralization Assays for A/H5N1 Virus Infections in Cultured Cells

Serial dilutions of antiserum were mixed with pandemic influenza viruses A/H1N1 (A/Osaka/47/2009 (H1N1) pdm) or avian A/H5N1 (A/Bogor 2/FKH-IPB/2008 (H5N1)) at 10^2 TCID₅₀ and incubated for 1 h at 37°C. Thereafter, these viral fluids were adsorbed onto MDCK monolayers for 1 h at 35°C, and then incubated in GIT media (Nakarai Tesque, Japan) containing low trypsin at 35°C. At 5 days post-incubation, the cultures were inspected for the cytopathic effect (CPE): the neutralizing titer, expressed as the reciprocal of antiserum dilution at which virus growth is 50% inhibited, was calculated by the number of virus-negative wells and the serum dilution according to the report by Reed *et al.* [21].

2.6. Experimental Challenge of Chickens with A/H5N1 and Antiserum Administration

Specific-pathogen-free (SPF) chicks were housed under controlled conditions in a BSL3 laboratory and received food and water *ad libitum*. At 10 days old, the birds were inoculated intranasally with A/H5N1 (A/Bogor 2/FKH-IPB/2008 (H5N1)) virus (10^5 TCID₅₀/ml) [22] [23] [24]. Some chicks were injected intramuscularly with antiserum (8, 400, 2000 mg/kg body weight) at 2 h prior to in-

fection, and all birds were housed for another 3 days. A higher volume of preimmune serum (2000 mg/kg) was administered to some birds as controls. At least 10 birds were prepared for each volume of the sera. At 3 days after inoculation, the dead birds were counted, and the survival rate was scored in each group (surviving birds/all birds examined). The survivors were sacrificed with pentobarbital sodium solution, and the tracheae and lungs were removed and immersed in 10% neutral buffered formalin for further histopathology and immunohistochemistry studies.

For the histopathology studies, paraffin sections were cut at 3- μ m with a microtome and stained with hematoxylin and eosin (H & E) per routine procedures and then observed under a light microscope.

All of the animal experiments were performed in accordance with the guidelines for studies with laboratory animals of the Kyoto Prefectural University Experimental Animal Committee.

2.7. Immunohistochemistry for Viral Antigens

Tracheae and lungs were fixed in buffered formalin and washed in PBS. The organs were soaked in 30% sucrose in PBS overnight. The organ pieces were mounted in a compound, frozen and cut into 20- μ m sections with a cryostat. The frozen sections were attached to glass slides and air-dried at room temperature. After being washed in PBS, the samples were incubated with a FITC-conjugated ostrich IgY against A/H5N1 viruses (1:1000) at 4°C overnight. Finally, they were mounted with glycerol following sufficient washing with PBS, and specific signals for viral antigens were examined under a fluorescent microscope [24].

3. Results

3.1. Generation of Chicken Antiserum against A/H5N1 Virus

The immunoreactivities of antisera against A/H5N1 virus were estimated by ELISA. The titers for A/H5N1 were dramatically increased in the serum at four weeks after the initial immunization (Table 1). In contrast, the antisera bound only slightly to A/H1N1 viral antigen. These findings indicate that specific antibodies were generated and transmitted into the bloodstream.

3.2. Inhibition of Hemaggregation Activities of A/H5N1 by Chicken Antiserum

First, the HA activities of the pandemic influenza A/H1N1 and avian A/H5N1 viruses were estimated using erythrocytes, since the viral strains in this study originated from sporadic cases of infection and their characteristics have not yet been clarified. Fortunately, both strains caused strong aggregations of erythrocytes. The highest dilutions of viral fluids showing hemaggregation were scored as a single HA unit, and both strains were used for further HI testing at 8 HAU. Hemaggregation by A/H5N1 virus was dramatically inhibited by the antiserum whereas that by A/H1N1 was not inhibited (Table 2).

Table 1. Binding activity of chicken antiserum by immunization with Avian Influenza virus A/H5N1.

Serum generated from adult chicken immunized with A/H5N1 antigens	Antibody titer against indicated influenza virus antigens (ELISA)	
	pandemic A/H1N1	Avian A/H5N1
antiserum	16	6400

The antibody titer against pandemic influenza A/H1N1 (A/Osaka/47/2009 (H1N1) pdm) and avian A/H5N1 (A/Bogor 2/FKH-IPB/2008 (H5N1)) viruses was estimated by ELISA. At 4 weeks post-immunization, the ELISA titers against A/H5N1 were clearly increased in chicken antiserum. In contrast, the reactivity against A/H1N1 virus did not increase in the serum. The ELISA titers were defined as the reciprocal of the highest dilution of antiserum that produced an ELISA signal twice as intense as the signal from equivalently diluted preimmune serum.

Table 2. Inhibitory activity of ostrich IgY on hemaggregations by Avian influenza virus A/H5N1.

virus	HI titer of ostrich IgY
pandemic A/H1N1	16
Avian A/H5N1	128

Purified erythrocytes were reacted with a pandemic influenza virus A/H1N1 (A/Osaka/47/2009 (H1N1) pdm) or an avian influenza virus A/H5N1 (A/Bogor 2/FKH-IPB/2008 (H5N1)) after incubation with antisera. The activity of antisera against each of the viruses was represented as HI titer ratios (HA titer with preimmune serum/HA titer with antiserum), with higher HI titers indicating a strong inhibitory activity of the antiserum against aggregation by the viruses. Note that the antiserum against A/H5N1 virus shows inhibitory activities on the hemaggregation by A/H5N1 virus.

3.3. Neutralization Assays for A/H5N1 Virus Infection in Culture Cells

The pandemic influenza virus A/H1N1 and avian influenza virus A/H5N1 were reacted with antiserum followed by inoculation into MDCK cells. As shown in **Table 3**, the cytopathic effects of MDCK cells infected with A/H5N1 were inhibited by the antiserum: the serum volume at 50% inhibition of A/H5N1 virus infection was much lower than that of A/H1N1 infection. These findings indicated that antiserum had neutralization activity against infection of A/H5N1 virus.

3.4. Effects of Antiserum on A/H5N1-Infected Chickens

The experimental challenge in living SPF chickens (10 days old) with A/H5N1 (A/Bogor 2/FKH-IPB/2008 (H5N1)) caused high lethality among birds (100%) within three days post-infection, indicating that this virus was a highly virulent type. Of note, all birds showed only slight symptoms, including dehydration and depression, followed by sudden death. In addition, all birds injected with preimmune serum died within three days after viral infection (**Figure 2**). A histopathological study of the infected chickens showed typical avian flu findings of acute inflammation accompanied by heterophilic infiltration, hemorrhage, edema and severe congestion in various organs, mainly in the pulmonary tissues, including the trachea and lung (**Figure 3**). The respiratory sections of infected birds showed slight-to-moderate inflammation with epithelial necrosis and

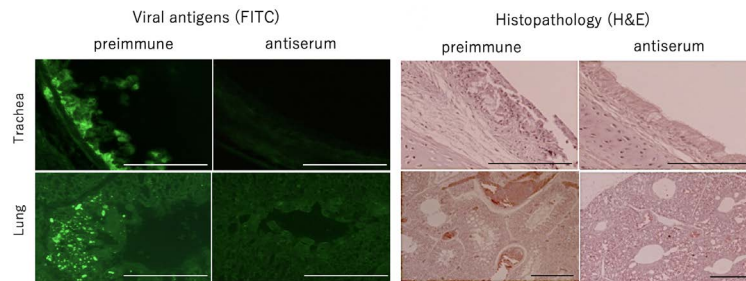


Figure 2. Effects of antiserum on the survival of A/H5N1-infected chicks. The chicks were intranasally challenged with A/H5N1 virus after being injected with antiserum. The dead chicks were counted at three days post-inoculation, and the survival rate was calculated. With preimmune serum, all of the infected birds died within three days. However, the number of survivors was dramatically increased by antiserum injections at over 0.08 mL/kg. Accordingly, the death of A/H5N1-infected chicks was completely inhibited by the administration of antiserum.

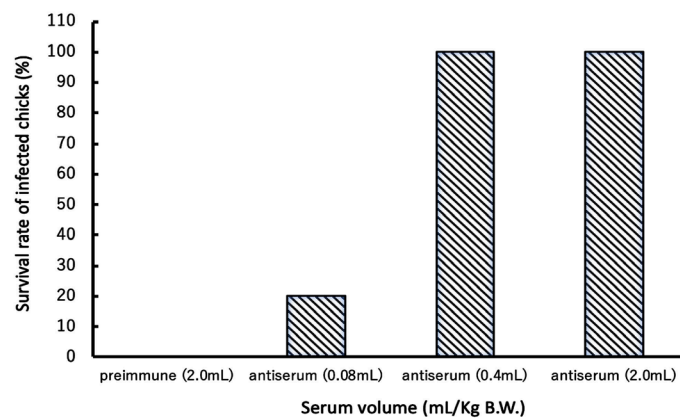


Figure 3. Trachea and pulmonary histopathology of A/H5N1-infected chickens injected with antiserum. Sections of A/H5N1-infected chick lung at three days post-inoculation with preimmune or antiserum were subjected to an immunofluorescent examination. Viral antigens were found in the tracheal epithelial cells and in pulmonary cells of A/H5N1 infected chicks injected with preimmune serum. In contrast, viral antigens were scarcely detected in either trachea or pulmonary tissues of infected birds injected with antiserum. Histopathologically, severe necrosis, heterophilic infiltration, hemorrhaging, edema and mucosal exudation were seen in the tracheal epithelium, interstitium, and parabronchial cavities in the lungs of infected chickens with preimmune serum, whereas pathological lesions were scarcely found in the organs of birds injected with antiserum. Bars, 200 μ m.

Table 3. Neutralizing activities of chicken antiserum against Avian influenza virus A/H5N1.

Serum	Neutralizing titers (50% inhibition) (μ L/1mL)	
	Pandemic A/H1N1	Avian A/H5N1
Preimmune serum	>225	>550
Antiserum	112.5	16

The neutralization assays were performed using MDCK infected with pandemic influenza A/H1N1 virus (A/Osaka/47/2009 (H1N1) pdm) or avian A/H5N1 virus (A/Bogor 2/FKH-IPB/2008 (H5N1)). The neutralizing titers are indicated as the mean of 50% inhibition on CPE at 5-days post infection.

heterophilic infiltration in the interstitium and parabronchial cavities. Severe hemorrhaging and congestion, accompanied by edema and mucosal exudates, were predominately seen. The viral antigens were found in the tracheal epithelial cells and pulmonary tissues of infected chicks at three days post-inoculation.

The antiserum was directly administered to A/H5N1-infected chicks to confirm the therapeutic and protective effects on avian flu infections. Interestingly, the survival rate of the infected birds was dramatically increased by an injection of antiserum (Figure 2). In addition, the pathological reactions in the infected tracheae and lungs in infected chicks were decreased by the injection of antiserum (Figure 3). The edema, hemorrhaging, congestion and mucosal exudation were clearly inhibited in the lesions. Viral antigens were scarcely found in the trachea and pulmonary tissues. These findings were consistent with results showing that the mortality of A/H5N1 chicks was decreased by antiserum administration. Accordingly, antiserum injection at a high volume was able to rescue all birds from death following A/H5N1 infection.

4. Discussion

The majority of A/H5N1 avian flu cases have been reported in southeast and east Asia. Once an outbreak is detected, local authorities often order a mass slaughter of domestic fowls infected or suspected of being infected. In this study, we proved that anti-sera against avian influenza virus can prevent infection in other chickens. Antiserum with a high neutralizing activity was found to be able to prevent chicken death via intramuscular injection of a very slight amount (40 μ L/bird). The virus used in this study was a virus strain isolated at a poultry farm in Indonesia and was a highly pathogenic virus [22] [23]. Nevertheless, a 100% survival rate of infected chickens was obtained by inoculation with antisera.

There are two key patterns in the suppression of influenza virus infection. The first is to block the HA antigen necessary for the virus to adsorb to the receptor of the host cell membrane. Influenza vaccine for humans contains HA proteins as antigens; anti-HA antibody is actively produced in the body, so virus infection can be prevented [25] [26]. The second is to suppress the neuraminidase necessary for shedding of viruses propagated in host cells. Oseltamivir and Zanamivir are neuraminidase inhibitors and are widely used as therapeutics for influenza [27] [28].

In poultry, some countries use vaccines for protection against avian influenza [9]. Usually, the inactivated virus body is used as an antigen instead of HA, but the infection prevention effect of this approach is unclear. Neuraminidase inhibitors are not used to prevent and treat influenza in domestic fowls. In a clean country, avian influenza vaccines are not used in poultry in order to prevent the misdiagnosis of natural infection and the opportunistic infection of viruses [9] [16]. We, therefore, expect that inoculation of poultry with antisera will be effective for preventing avian influenza. Antibodies that inhibit HA antigen and neuraminidase are thought to be present in these antisera.

In the future, we will conduct a large-scale experiment at a poultry farm and examine the usefulness of antiserum administration in cases of natural infection. The present findings suggest that the administration of antisera and antibodies may contribute to the prevention of infection, even during a pandemic of avian influenza transmitted from human to human.

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Conflicts of Interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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