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Efficient capture of infectious H5 avian influenza virus utilizing magnetic beads coated with anionic polymer

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ABSTRACT

The possible emergence of a pandemic influenza virus from the avian influenza virus (AIV) has become a serious threat. The isolation of viruses will be crucial for further virological analysis and the development of vaccines. However, currently, there is no simple method for facilitating the isolation of infectious AIV. Here, we have developed a simple method of capturing AIV using anionic magnetic beads. The method employed the capture of AIV (H5N1, H5N2, and H5N3) from liquid samples such as allantoic fluid (AF) and cell culture medium (CM) using magnetic beads coated with an anionic polymer, poly(methyl vinyl ether-maleic anhydride). After their incubation with AIV-containing samples, the magnetic beads were separated from the supernatant by applying a magnetic field. The absorption of AIV on the beads was confirmed by immunochromatography and an enzyme-linked immunosorbent assay, which indicated the presence of hemagglutinin, neuraminidase, and nucleoprotein of AIV. Furthermore, the infectivity in chicken eggs of AIV captured by magnetic beads was similar to that of the starting materials. The capture of AIV using magnetic beads coated with anionic polymers will contribute to the sufficient recovery of infectious AIV and approach for potential pandemic influenza viruses.

Strains of avian influenza virus (AIV) are classified as highly pathogenic (HPAIV) or low pathogenic (LPAIV), based on their pathogenicity in poultry [1]. Not all strains of the H5 and H7 subtypes are highly pathogenic, but it is H5 and H7 HPAIV which often cause systemic infections in poultry [2–4]. Among hundreds of AIV strains, only four, H5N1, H7N3, H7N7 and H9N2, are known to have caused human infections [1]. Although human infections with the latter three viruses have resulted in mild symptoms and very little severe illness, H5N1 AIV is highly pathogenic not only to poultry but also to humans [1].

The World Health Organization (WHO) reports that there have been 385 human cases of H5N1 HPAI infection and 243 deaths as of 19 June 2008 [5], a mortality rate of more than 60%. The human cases have been reported in 15 countries: Cambodia, China, Indonesia, Thailand, Turkey, Lao People's Democratic Republic, Myanmar, Bangladesh, Pakistan, Iraq, Djibouti, Azerbaijan, Egypt, Nigeria, and Vietnam [6]. These cases have coincided with outbreaks of HPAIV H5N1 in poultry. H5N1 differs from the general human influenza viruses (A/H1N1, A/H3N2, and type B) where it infects and proliferates. The latter viruses infect and proliferate in human epithelial cells of the upper respiratory tract, while H5N1 targets type II alveolar epithelial cells of the lower respiratory tract [7].

In terms of diagnosis, early on it is difficult to distinguish H5N1 AIV infections from other respiratory diseases such as infections of H3N2, H1N1, adenovirus, respiratory syncytial virus (RSV), hemolytic streptococcus, and metapneumovirus [8]. More importantly, early treatment (<12 h after clinical onset) is essential for the effective oral administration of oseltamivir [9]. Several methods for detecting H5N1 AIV have been developed such as the enzyme-linked immunosorbent assay (ELISA) [10], the immunoblot [10], the polymerase chain reaction (PCR) [11–13], and immunochromatography [14]. Immunochromatography is useful for the rapid diagnostic assay of H5N1 AIV infections. Furthermore, the threat of the emergence of pandemic influenza viruses necessitates early detection and the isolation of potential pandemic viruses for preventing the rapid spread of infection by developing vaccines. However, these methods do not enable us to isolate infectious particles because the viruses are inactivated in the assay process. Infection of embryonated eggs or cell culture medium is required for isolation, and a concentration step is important for the efficient and early isolation of viruses. Meanwhile, methods of concentration such as polyethylene glycol (PEG) precipitation and ultracentrifugation often decrease the infectivity of viruses [15,16]. One approach is to use magnetic beads to concentrate AIV without reducing its infectivity.

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Here, we report that magnetic beads coated with an anionic polymer, poly(methyl vinyl ether-maleic anhydride) [poly(MVE-MA)], are useful for the capture of infectious AIV. The potential of this method is discussed.

Materials and methods

Reagents. Unless otherwise specified, chemical reagents were obtained from Sigma (St. Louis, MO) or Wako Pure Chemical Industries (Osaka, Japan). The anionic magnetic beads used in the present study are monozides 300 nm in diameter (reducing sedimentation and offering a wide binding surface) with a high ferrite content (allowing separation under a magnetic field) and prepared by the grafting of poly(MVE-MA) in a dimethyl sulfoxide (DMSO)/Phosphate buffer 5/95 solution for 3 h at 37 °C [17]. These beads are available commercially as Viro-adembeads (Ademtech, Pessac, France).

Virus. HPAIV [A/crow/Kyoto/53/2004 (H5N1)] and LPAIV [A/duck/Hong Kong/342/78 (H5N2), A/duck/Hong Kong/820/80 (H5N3)] were used.

AIV capture. Viral capture was performed using the company's instructions (Ademtech). Briefly, after two washes with binding buffer, anionic magnetic beads (50 μl) were further washed twice with phosphate-buffered saline (PBS). Then, 50 μl of sample such as allantoic fluid (AF) from a mock-infected egg or egg infected with AIV H5N1, H5N2, or H5N3, culture medium (CM) of primary porcine alveolar epithelial cells mock-infected or infected with AIV H5N1, H5N2, or H5N3, or virus purified from CM by sucrose gradient ultracentrifugation, was diluted with 500 μl of PBS and exposed to the washed magnetic beads for 20 min at room temperature. Tubes containing the magnetic beads were set under a magnetic field. The beads were subjected to separation by discarding the supernatant, and washed three times in PBS. The washed beads were resuspended with PBS (50 μl) and subjected to immunochromatography, ELISA, or egg infection.

ELISA. Each fraction (50 μl) was diluted with 500 μl of PBS and subjected to ELISA (50 μl/well). ELISA using antibodies against hemagglutinin (HA), the internal region of neuraminidase [NA(IN)], and the C-terminal region of neuraminidase [NA(CT)] was performed with an Avian Flu (H5N1) Detection Set (AnaSpec Inc., San Jose, CA). HA and NA concentrations were compared with concentrations of reference peptides corresponding to epitopes of each antibody and estimated using a standard curve of each reference peptide versus absorbance at 450 nm.

Immunochromatography. Immunochromatography for the influenza A and B virus nucleoprotein (NP) was performed using an Epsrine Influenza A & B-N (Fujirebio Inc., Tokyo, Japan).

Egg infection. Infectivity was determined with 100-μl inoculums in chicken eggs using standard methods. The inoculation was conducted in 11-day-old embryonated eggs. The beads fraction, supernatant after incubation, and whole sample containing the same quantity of start material as the beads fraction (each 50 μl) were diluted with 500 μl of PBS and inoculated into eggs. Chicken AF was collected from the eggs 16 h post-inoculation and subjected to immunochromatography.

Results and discussion

To examine the capacity of magnetic beads to capture AIV, immunochromatography, ELISA, and the inoculation of eggs were performed. Immunochromatography showed that HPAIV H5N1 NP was predominantly detected in the beads fraction (BD) at similar levels to the total sample containing the same quantity of start materials as BD (TL) but not in the supernatants (SP) using AF, CM, and purified virus (PV) samples infected with H5N1 (Fig. 1). These results suggest that the method is applicable to AIV in both AF and CM. However, capture efficiency was slightly lower in CM than in AF and PV possibly due to non-specific binding of serum proteins.

Next, we examined the efficiency of capture by quantitative ELISA using antibodies against HA, the internal region of NA, and the C-terminal region of NA (Fig. 2). ELISA showed that H5N1 was recovered with anionic magnetic beads from H5N1-infected AF (beads fraction, BD) at a similar level to H5N1 in sample containing the same quantity of AF as BD (TL), whereas H5N1 was undetectable in the supernatant after incubation (SP) from H5N1- and mock-infected AF and the beads fraction (BD) from mock-infected AF, suggesting that most of the H5N1 having HA and NA was efficiently captured by the beads.

Second, we examined whether this method is applicable to other AIVs including LPAIV H5N2 and H5N3. Immunochromatography for NP showed that not only H5N1 but also H5N2 and H5N3 could be recovered in the beads fraction (BD) from the culture medium of AIV-infected primary porcine alveolar epithelial cells (Fig. 3).

Finally, we examined the infectivity of viruses recovered with magnetic beads using eggs. The total sample fraction (TL) and beads fraction (BD) from H5N1 AIV-infected AF exhibited a 100% (5/5) infection rate in embryonated eggs. In contrast, the supernatant from H5N1 AIV-infected AF showed no bands on immunochromatography for NP in 60% (3/5) of eggs but a very weak band in the other eggs (Fig. 4). No bands for AIV NP were detected in mock-infected AFs after the inoculation. These results show that infectious H5N1 AIV was present not only in the total sample fraction (TL) but also in the beads fraction (BD) at similar levels. Taken together, they support that infectious H5N1 AIV is efficiently captured by anionic magnetic beads, suggesting that the binding of these beads to AIV does not reduce its infectivity.

Factors limiting the development of methods to concentrate viruses include compatibility with current methods of detection and convenience. Furthermore, the recovery of infectious particles is required for isolation and further virological analysis. Several attempts at concentrating AIV to enhance sensitivity have been made [18,19]. Ultracentrifugation is a well-known method, but time-consuming and can increase the false-positive rate when combined with PCR [20]. PEG precipitation is simple and easy to perform, but the PEG inhibits PCR [21]. Furthermore, both ultracentrifugation

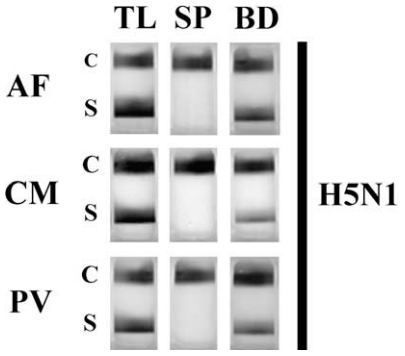

Fig. 1. Detection of nucleoprotein (NP) in the highly pathogenic avian influenza virus (HPAIV) H5N1 absorbed on anionic magnetic beads from allantoic fluid (AF), cell culture medium (CM) and purified virus (PV). AF, CM, and PV containing HPAIV H5N1 were diluted with phosphate-buffered saline (PBS) and incubated with anionic magnetic beads. Esprine Influenza A and B-N (Fujirebio Inc., Tokyo, Japan) was used for the detection of influenza virus nucleoprotein (NP) by immunochromatography. Results of samples (S) were interpreted on the basis of the presence and absence of a line included in the kit as a positive control (C). Samples were divided into three categories; a beads fraction (BD), supernatant after the incubation (SP), and total sample containing the same quantity of start material (AF, CM, or PV) as BD (TL). They were solubilized with lysis buffer and subjected to immunochromatography.

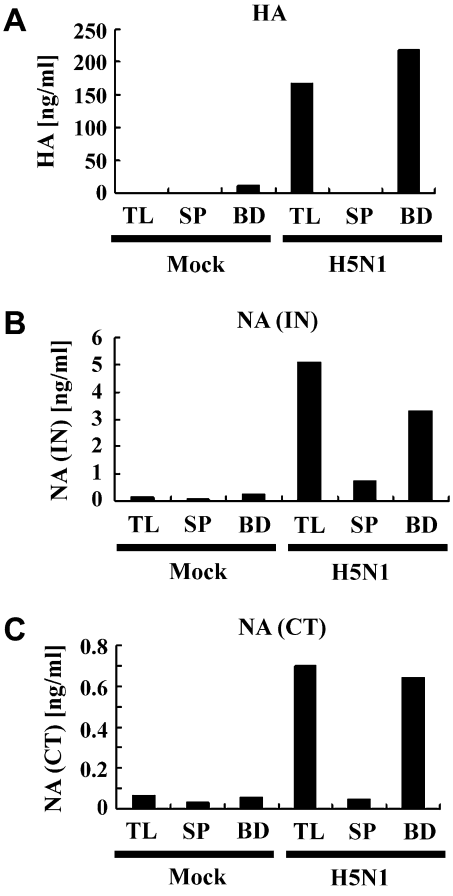

Fig. 2. Quantitative analysis of HPAIV H5N1 absorbed on anionic magnetic beads. HPAIV H5N1 in AF before and after absorption on anionic magnetic beads was quantitatively analyzed by enzyme-linked immunosorbent assay (ELISA) using an Avian Flu (H5N1) Detection Set (AnaSpec Inc., San Jose, CA). Samples from mock- and H5N1-infected AFs were divided into three categories; a beads fraction (BD), supernatant after the incubation (SP), and sample containing the same quantity of start material as BD (TL). The quantities of hemagglutinin (HA), neuraminidase (NA) detected by antibody against the internal region [NA(IN)], and NA detected by antibody against the C-terminal region [NA(CT)] in each fraction were compared.

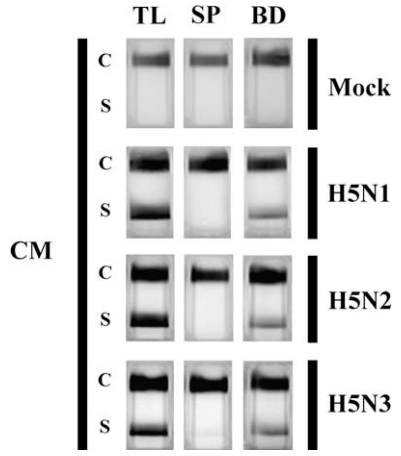

Fig. 3. Detection of NP in HPAIV (H5N1) and low pathogenic avian influenza virus (LPAIV) (H5N2 and H5N3) absorbed on anionic magnetic beads. HPAIV H5N1 and LPAIV H5N2 and H5N3 in the culture medium (CM) of AIV-infected primary porcine alveolar epithelial cells were diluted with PBS and subjected to incubation with anionic magnetic beads. Esprine Influenza A and B-N (Fujirebio Inc.) was used for the detection of influenza NP by immunochromatography. Results of samples (S) were interpreted on the basis of the presence and absence of a line included in the kit as a positive control (C). Samples were divided into three categories; a beads fraction (BD), supernatant after the incubation (SP), and total sample containing the same quantity of start meterial as BD (TL). They were solubilized with lysis buffer and subjected to immunochromatography.

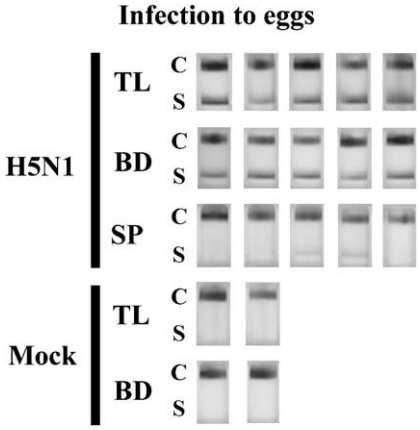

Fig. 4. Infections by HPAIV H5N1 recovered with anionic magnetic beads in embryonated eggs. HPAIV H5N1 in infected AF and mock-infected AF was captured by anionic magnetic beads. The beads fraction (BD), supernatant after incubation (SP), and sample containing the same quantity of AF as BD (TL) were inoculated into eggs and incubation carried out as described in Materials and Methods. The resultant AFs were analyzed by immunochromatography using Esprine Influenza A and B-N (Fujirebio Inc.). Results of samples (S) were interpreted on the basis of the presence and absence of a line included in the kit as a positive control (C).

and PEG precipitation often decrease the infectivity of a virus after its concentration. It must be emphasized that any method to concentrate a virus should be simple, because the possibility of loss and cross-contamination among samples increases with multiple steps. Capture by magnetic beads is therefore a promising approach. One method is to use magnetic beads coated with molecules which efficiently bind viral particles without decreasing their infectivity.

There are several methods for concentration using magnetic beads coated with an antibody for a specific virus and polymer such as polyethyleneimine (PEI) for simian virus 40 (SV40) [22], herpes simplex virus type 1 (HSV-1) [22], Sindbis virus [22], vesicular stomatitis virus (VSV) [22], amphotropic murine leukemia virus [23], poliovirus, hepatitis A virus (HAV) [24], hepatitis B virus (HBV) [24], hepatitis C virus (HCV) [24], and cytomegalovirus (CMV) [25] or sulfonated magnetic (SO-magnetic) beads in the presence of divalent cations for cytomegalovirus [25], Sindbis virus [25], poliovirus [25], and porcine parvovirus [25]. Although PEI-conjugated magnetic or SO-magnetic beads were successfully used for the concentration of several viruses, the recovery of infectious particles has not been described. Therefore, this is the first study to clearly show that poly(MVE-MA)-coated magnetic beads can be used for the capture of an infectious virus, AIV. However, it remains unclear how the beads bind to AIV. As HBV having envelope protein could not be captured by anionic magnetic beads (Sakudo, unpublished result), the binding ability does not depend on the presence of envelope protein. One explanation is that electrostatic, hydrophilic and hydrophobic interactions, along with steric phenomena, are involved. Anionic magnetic beads are negatively charged and modification of the spatial organization of the beads could decrease their binding capacity [17]. Charge density and steric spatial organization may provide some information on the binding mechanism. Regretfully, anionic magnetic beads appear to bind blood components such as albumin (Sakudo, unpublished results). Therefore, this method can be used for concentration but not puri-

fication of AIV. However, some modification affecting charge density and surface chemistry may reduce the non-specific binding.

In conclusion, we demonstrated that magnetic beads coated with an anionic polymer are useful for the capture of AIV. In the captured AIV, the presence of HA, NA, and NP was confirmed by ELISA and immunochromatography. Furthermore, the AIV recovered using the beads had fully preserved infectious activity in eggs. These results suggest that this viral capture method can be used in combination with conventional methods of detection methods. Efficient monitoring of infectious AIV isolated from humans and poultry may provide the opportunity to detect a potential pandemic virus early enough to eliminate the virus at its source and prevent a pandemic by developing vaccines. We have confirmed the usefulness of this viral capture method for human immunodeficiency virus (HIV), influenza A virus (H1N1, H3N2, H5N1, H5N2, and H5N3), influenza B virus, and RSV (Sakudo, unpublished result). Revealing the broadness of the applicability of this capture method may also contribute to general public health, especially by enhancing the detection and isolation of viruses.

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