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Production and characterization of monoclonal antibodies against an avian influenza virus (H9N2)

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Abstract

In this report, fifteen monoclonal antibodies (MAbs) against an avian influenza virus (H9N2 subtype) were newly produced and characterized. These MAbs proved to react to the epitopes of nucleocapsid protein (NP), hemagglutinin (HA), neuraminidase (NA) and non-structural protein 1 (NS1) of Korean H9N2 strain, respectively. Two HA-specific MAbs showed the ability to inhibit the hemagglutination activity of H9N2 subtype avian influenza virus when tested by hemagglutination inhibition (HI) assay. All MAbs did not cross-react with other avian-origin viruses (Newcastle disease virus, infectious bursal disease virus, infectious bronchitis virus and avian rotavirus) by immunofluorescence test or enzyme-linked immunosorbent assay. The MAbs produced in this study could be useful as the materials for diagnostics and therapeutics against Korean-lineage H9N2 virus infections.

Key words : Avian influenza virus, H9N2, Monoclonal antibody

INTRODUCTION

Avian influenza virus (AIV) belongs to the genus Influenza A virus under the family *Orthomyxoviridae* and are spherical, 80∼120 nm in diameter. The AIV genome is negative-sense, single-stranded RNA and consists of 8 gene segments encoding 11 proteins (Suarez, 2005). These viruses were classified into subtypes on the basis of serological differences between the two surface glycoproteins called hemagglutinin (HA) and neuraminidase (NA). To date, total 16 HA and 9 NA subtypes of AIVs have been isolated from avian species. Among several subtypes, H9N2 subtype viruses have the main role for the causative agents of low pathogenic avian influenza (LPAI) infections in domestic chicken flocks (Lee and Song, 2013).

Several groups of H9N2 LPAI virus (LPAIV) have been prevalent in Eurasian countries, including the G1, Y-280, BJ94, G9 and Korean lineages (Guan et al, 1999; Xu et al, 2007). Since the first LPAI outbreak in 1996, distinct Korean lineage viruses have become established and continuously occurred in Korea (Lee et al, 2000; Lee et al, 2007). To control LPAI outbreaks, the oil-based inactivated H9N2 vaccinations (A/chicken/Korea/01310/2001 H9N2 LPAIV strain) were approved by Korean animal quarantine agency, and employed widely in domestic breeder and laying chickens flocks (Choi et al, 2008). Recently, Y280 and G1-like H9N2 viruses have been isolated from humans showing influenza-like symptoms (Butt et al, 2005; Huang et al, 2015).

LPAI infections represented no significant pathognomonic signs, making it difficult for poultry veterinarians

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to diagnose LPAI infection in the field. Therefore, the rapid and accurate diagnostics should be needed for the identification of LPAI infections (Gavin and Thomson, 2004). However, commercial AIV diagnostic kits could have lower sensitivity to LPAIVs due to the constant emergence of AIV mutants (Carrat and Flahault, 2007; Chen and Holmes, 2006). There were no commercial H9N2 vaccines available in humans although H9N2 human infections have been continuously reported (Butt et al, 2005; Huang et al, 2015). Monoclonal antibodies (MAbs) have been the choices for new materials of diagnostics and therapeutics against new emerging infectious diseases (Nguyen et al, 2017; Qiu et al, 2014).

In the present study, we newly produced and characterized total 15 monoclonal antibodies (MAbs) against for nucleoprotein (NP), HA, NA and non-structural 1 (NS1) proteins of a Korean H9N2 isolate. The characteristics of these MAbs were successfully evaluated by indirect immunofluorescence assay (IFA), hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assay.

MATERIALS AND METHODS

Viruses and recombinant AIV proteins

H9N2 LPAI field virus (A/Chicken/Cheongju/ADL0401) was originally isolated from Korean laying chickens flocks showing decreased egg production and moderate high mortality. The virus was grown in the allantoic cavities (AC) of 9- to 11-day-old specific pathogen free (SPF) chicken embryos and collected aseptically at 3 day post inoculation (dpi). The harvested AC fluids were purified and concentrated by ultracentrifugation using the sucrose density gradient method as previously described (Hiernaux and Bona, 1982). Recombinant NP, HA, NA and NS1 proteins of AIV were already expressed from baculovirus vectors in our previous report (Le Van Phan, 2006).

Production of MAbs

Total 15 MAbs were produced by immunizing BALB/C

mice with the purified AIV and recombinant NS1 proteins three times as follows. In brief, the mice were inoculated with 0.1 mL ADL0401 strain and recombinant NS1 protein emulsified with the complete Freund's adjuvants (Sigma-Aldrich, USA) at the first immunization, and then the same dose of inocula were given to mice twice with incomplete Freund's adjuvants (Sigma-Aldrich, USA) at the second and third immunization. After 2 weeks, lymphocytes from the spleens and lymph nodes of mice were harvested and fused with SP/2.O myeloma cells as previously described (Köhler and Milstein, 1975). Fused cells were cultured with hypoxanthine-aminopterin-thymidine medium for 14 days and MAbs produced by hybridomas were identified by IFA test. Positive cells were selected and subcloned three times by limiting dilution procedures. Isotyping of cloned MAbs was performed using goat anti-mouse IgG (Sigma-Aldrich, USA).

Hemagglutination inhibition (HI) assay

HI assay was performed using chicken RBC (cRBC) in V-bottom 96-well microplates according to a general method recommended by the World Health Organization (WHO). Briefly, the supernatant containing MAbs were serially diluted 2-fold with 0.1M phosphate buffered saline (PBS, pH 7.2). The concentration of LPAI H9N2 virus was adjusted to 4 hemagglutination unit (HAU). 25 μL LPAI virus (4HAU) was added onto all wells, and mixed gently with MAbs. After incubation at room temperature for 20 min, 25 μL 1% chicken erythrocytes in PBS was added to all wells, and then incubated at room temperature for 40 minutes. Finally, the results were interpreted and recorded.

Neuraminidase inhibition (NI) assay

Neuraminidase inhibition (NI) assay was performed in V-bottom 96-well microplates by the procedure recommended by the World Health Organization (Aymard-Henry et al, 1973). Briefly, harvested AC fluids was diluted 3-fold with 0.1M PBS. MAbs were serially diluted (2-fold steps) in 0.1M PBS and then mixed with 25 μL of the viruses. These mixtures were added to each well

and incubated at room temperature for 1 hour. 25 μL of fetuin solutions (0.05M PBS, 1.25% fetuin, pH 5.9) were added to all wells and incubated at 37℃ for 3 hours. 25 μL of 0.125N H_2SO_4 containing 0.025M sodium periodate was added to all wells and incubated at room temperature for 20 minutes. 25 μL of 0.5N HCl containing 2% sodium arsenite and 100 μL of 0.1M thiobarbituric acid (pH 9.0) were added to all wells, and then the results were recorded after incubation at 56℃ for 30 minutes.

Identification and titration of MAbs in IFA test

The IFA test was performed to identify MAbs production. Briefly, Sf9 cells were infected with recombinant baculoviruses containing each NP, HA, NA and NS1 gene. After cytopathic effect (CPE) formations in Sf9 cells, the cells were fixed for 10 minutes with 80% acetone. 100 μL of hybridoma culture supernatants were inoculated onto the Sf9 cell monolayers in 96-well plates and stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson immune Research Laboratories, USA). The IFA titers of MAbs derived from ascites fluids were determined as follows. Briefly, Madin-Darby canine kidney (MDCK) cells were raised in 96-well microplates and infected with H9N2 virus as described previously (Glikmann et al, 1995). After cytopathic effect (CPE) formation, the cells were fixed with 80% acetone for 10 minutes. 100 μL dilutions of hybridoma culture fluids were inoculated onto the Sf9 cell monolayers in 96-well plates, and stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson immune Research Laboratories, USA).

Cross−reactivities of MAbs to other subtypes of LPAIVs

The MAbs were examined for the cross-reactivities to other 10 subtypes LPAIVs including H3N8, H4N1, H4N6, H4N8, H5N2, H5N3, H6N2, H6N8, H7N7 and H9N1 viruses using IFA test. The test was performed in MDCK cells according to the procedures as described above.

Table 1. Characterization of monoclonal antibodies (MAbs) against avian influenza virus (AIV) H9N2

MAb	Isotypes	Protein specificity[a]	Antibody titers against H9N2		
			IFA	HI	NI
5E7	G2a	NP	>10,240	<2	<2
5D7	G2a	NP	>10,240	<2	<2
6B5	G2a	NP	1,280	<2	<2
17E1	G1	HA	2,560	<2	<2
18E10	G3	HA	10,240	64	<2
20B6	M	HA	2,560	<2	<2
23D12	G1	HA	5,120	128	<2
24F7	G1	HA	10,240	<2	<2
8H9	G2a	NA	160	<2	<2
9D7	G1	NA	320	<2	<2
1A4	G1	NS1	10,240	<2	<2
1F12	G1	NS1	2,560	<2	<2
2E1	G1	NS1	5,120	<2	<2
3B9	G1	NS1	>10,240	<2	<2
5E6	G1	NS1	5,120	<2	<2
3F2	G1	M1	>10,240	<2	<2

[a]Reactivities of MAbs to each viral protein were determined using IFA tests based on each recombinant NP, HA, NA and NS1 protein expressed in baculovirus express systems.
NP: Nucleocapsid, HA: hemagglutinin, NA: Neuraminidase, NS1: Nonstructural protein1, M1: Matrix protein, IFA: indirect fluorescence antibody test, HI: Hemagglutination inhibition test, NI: Neuraminidase inhibition test.

Cross−reactivities of MAbs to other non−AIV, avian−origin viruses

The cross-reactivities of the MAbs to other non-AI avian-origin viruses including Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), avian rotavirus (ARV) and infectious bronchitis virus (IBV) were evaluated using IFA and ELISA methods. Briefly, NDV, IBDV and ARV were examined for the reactivities to MAbs using IFA tests as described above. Also, the reactivities of MAbs to IBVs were evaluated using commercial IBV ELISA kits (IDEXX, USA) according to manufacturer's instructions.

RESULTS

Production and characterization of MAbs

Fifteen clones of hybridoma cells secreting the H9N2 subtype-specific MAbs were produced and characterized (Table 1). The IFA results showed the reactivity of MAbs to each protein of AIVs, respectively (Table 2). In brief, three NP-specific MAbs (5D7, 5E7, 6B5), five HA-specific MAbs (17E1, 18E10, 20B6, 23D12, 24F7),

two NA- specific MAbs (8H9, 9D7) and five NS1-specific MAbs (1A4, 1F12, 2E1, 3B9, 5E6) were finally confirmed. The two MAbs (18E10, 23D12) represented 64 and 128 HI titers, whereas NI activity was not observed in all MAbs (Table 1). The IFA titers of the MAbs ranged from 160 to over 10,240.

Cross−reactivities of MAbs to other subtypes of LPAIVs

The reactivities of MAbs to other subtypes of LPAIVs were examined using IFA test (Table 2). Six of 15 MAbs (5E7, 5D7, 6B5, 1A4, 1F12, 2E1) recognized all other subtypes AIVs (H3N8, H4N1, H4N6, H4N8, H5N2, H5N3, H6N2, H6N8, H7N7, H9N1) whereas two MAbs (8H9, 9D7) reacted to limited subtypes of AIVs (H5N2, H6N2). The MAbs, 24F7 and 5E6, reacted to two subtypes (H9N2, H9N1) and five subtypes (H3N8, H5N2, H5N3, H6N2, H9N1), respectively. The remaining four MAbs (17E1, 18E10, 20B6, 23D12) reacted to none of AIV subtypes except the subtype H9N2 virus.

Table 2. Reactivity patterns of AIV (H9N2)-specific monoclonal antibodies (MAbs) to other subtypes of avian influenza virus by IFA test

MAbs		Reaction with other AIV subtype									
Name	Protein Specificity[a]	H3N8	H4N1	H4N6	H4N8	H5N2	H5N3	H6N2	H6N8	H7N7	H9N1
5E7	NP	○	○	○	○	○	○	○	○	○	○
5D7	NP	○	○	○	○	○	○	○	○	○	○
6B5	NP	○	○	○	○	○	○	○	○	○	○
17E1	HA										
18E10	HA										
20B6	HA										
23D12	HA										
24F7	HA										○
8H9	NA					○		○			
9D7	NA					○		○			
1A4	NS1	○	○	○	○	○	○	○	○	○	○
1F12	NS1	○	○	○	○	○	○	○	○	○	○
2E1	NS1	○	○	○	○	○	○	○	○	○	○
3B9	NS1	○	○	○		○	○	○		○	
5E6	NS1	○				○	○	○			○

Circles indicate positive reactions in IFA tests.
[a]Specific proteins of H9N2 virus reacted to MAbs.

Cross−reactivities of MAbs to other non−AIV, avian−origin viruses

The MAbs did not cross-react to other avian-origin viruses (NDV, IBDV, IBV and ARV) in IFA or ELISA tests.

DISCUSSION

AI was one of the prevalent diseases in poultry industries. Despite broad eradication and vaccination policy, AI has been continuously occurred in Korea due to constant emergence of AIV variants (Lee et al, 2016). Therefore, new effective diagnostics and therapeutics should be developed against Korean AIVs. In this study, we produced and characterized total 15 MAbs against Korean lineage H9N2 LPAI field isolate.

The 15 MAbs specific for NP, HA, NA and NS1 proteins of H9N2 virus were successfully produced and confirmed using IFA test (Table 1). In Korea, distinct Korean lineage LPAIVs were prevalent in commercial chicken flocks (Lee et al, 2000; Lee et al, 2007). Therefore, we produced MAbs targeting viral proteins of a Korean field isolate to decrease the mismatches between MAbs and viral proteins. IFA results showed that total 6 NP- and NS1-specific MAbs reacted to all other subtypes of AIVs in this study. Non-structural viral proteins commonly shared higher amino acid sequence similarities among AIV subtypes compared to surface structural proteins, HA and NA. Therefore, these 6 MAbs could be used as new materials for AIV diagnostics such as ELISA and rapid immunochromatographic assay (RIA).

All 5 HA-specific MAbs reacted to H9N2 virus in IFA tests. Interestingly, only one MAb (24F7) have the ability to react to both H9N2 and H9N1 viruses. 4 MAbs specific for only H9N2 virus are supposed to bind to highly variable regions within HA proteins (Obenauer et al, 2006), making it difficult to be used as materials of diagnostics due to high limited reactivity to H9 viruses. Also, 2 NA-specific MAbs (8H9, 9D7) represented specific reactivity to all 3 N2 subtype AIVs (H9N2, H5N2 and H6N2). The combination of one H9-specific MAb (24F7) and two N2-specific MAbs (8H9, 9D7) could be used as the materials of diag-

nostics specific for Korean lineage H9N2 LPAIVs.

2 HA-specific MAbs (18E10, 23D12) significantly inhibited hemagglutination activity of H9N2 virus when tested by HI tests (Table 1). There was the high positive correlation between the titers of HI and viral neutralization tests (Truelove et al, 2016). Therefore, our 2 MAbs with significant HI activity could efficiently neutralize H9N2 viruses. Not only avian-origin H9N2 viruses caused respiratory distress and egg drops in chickens, but also this virus can cause influenza-like symptoms in humans (Butt et al, 2005; Huang et al, 2015). In previous reports, the significant increase in drugs resistance among AIVs was reported (Bright et al, 2005; Ison et al, 2006). Therefore, new therapeutic agents against H9N2 virus need to be developed especially human LPAIV infection. Recently, Z-Mapp comprising three chimeric MAbs against ebola virus was developed and used to treat ebola virus-infected patients in national disaster (Qiu et al, 2014). Our 2 MAbs showing significant HI titers could be the choices as antiviral agents in future H9N2 AIVs epidemics.

To summarize, total 15 MAbs specific for each viral protein of Korean H9N2 isolate were successfully produced and characterized. This report is the first to describe production of MAbs specific for Korean H9N2 LPAIV strain. These MAbs have potential to be widely used as valuable materials of efficient diagnostics and therapeutics. In further study, we should evaluate the efficacy of MAbs in animal experiments.

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