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Oral administration of live attenuated *Salmonella enterica* serovar Typhimurium expressing chicken interferon-α alleviates clinical signs caused by respiratory infection with avian influenza virus H9N2

Md Masudur Rahman [a], Erdenebileg Uyangaa [a], Young Woo Han [a], Seong Bum Kim [a], Jin Hyoung Kim [b], Jin Young Choi [a], Dong Jin Yoo [c], Jin Tae Hong [d], Sang-Bae Han [d], Bumseok Kim [a], Koanhoi Kim [e], Seong Kug Eo [a,*]

[a] College of Veterinary Medicine and Bio-Safety Research Institute, Chonbuk National University, Jeonju 561-756, Republic of Korea
[b] Department of Biology, College of Natural Science, Chonbuk National University, Jeonju 561-756, Republic of Korea
[c] Department of Hydrogen and Fuel Cells, Specialized Graduate School, Chonbuk National University, Jeonju 561-756, Republic of Korea
[d] College of Pharmacy and Medical Research Center, Chungbuk National University, 410 Seongbong, Heungduk, Cheongju, Chungbuk 361-763, Republic of Korea
[e] Department of Pharmacology, School of Medicine, Pusan National University, Busan 602-739, Republic of Korea

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ABSTRACT

Low pathogenic avian influenza (LPAI) H9N2 has attracted considerable attention due to severe commercial losses in the poultry industry. Furthermore, avian influenza virus (AIV) H9N2-infected chickens can be a reservoir for viral transmission to mammals including pigs and humans, complicating control of viral mutants. Chicken interferon-alpha (chIFN-α) may be useful as an exogenous antiviral agent to control AIV H9N2 infection. However, a superior vehicle for administration of chIFN-α is needed because of challenges of protein stability, production cost, and labor associated with mass administration. Presently, oral administration of single dose of attenuated *Salmonella enterica* serovar Typhimurium expressing chIFN-α alleviated clinical signs and histopathological changes caused by respiratory infection with AIV H9N2 and reduced the excretion of virus in cloacal swab samples. Similarly, chickens administered *S. enterica* serovar Typhimurium expressing chIFN-α showed inhibited replication of AIV H9N2 in several different tissues including trachea, lung, cecal tonsil, and brain. Furthermore, immune responses specific for challenged AIV H9N2 were enhanced in chickens administered *S. enterica* serovar Typhimurium expressing chIFN-α, as determined by hemagglutination inhibition assay of sera, proliferation and IFN-γ and interleukin-4 expression by AIV H9N2 antigen-stimulated peripheral blood mononuclear cells and splenocytes. Therefore, oral administration of *S. enterica* serovar Typhimurium expressing chIFN-α can successfully control clinical signs caused by respiratory infection with AIV H9N2, which provides valuable insight into the use of attenuated *Salmonella* vaccine as an oral delivery system of chIFN-α to prevent the replication of AIV H9N2 in respiratory tract.

1. Introduction

Avian influenza viruses (AIV) H9N2 have become panzootic in Asia during the past decade and have been isolated from terrestrial poultry worldwide (Cameron et al., 2000; Choi et al., 2004; Guo et al., 2000; Li et al., 2003). Since 1996 outbreak of low pathogenic avian influenza (LPAI) H9N2 (Lee et al., 2000), the virus has become endemic in Korea, especially in layer farms (Kwon et al., 2006; Lee et al., 2007) and has attracted considerable attention due to its rapid spread across Korean chicken farms with enhanced clinical severity and increasing

* Corresponding author. Tel.: +82 63 270 3882; fax: +82 63 270 3780.
E-mail address: vetvirus@chonbuk.ac.kr (S.K. Eo).

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mortality (up to 65% for leghorn layer chickens) (Lee et al., 2007). Of additional concern, AIV H9N2-infected chickens can serve as reservoir host and transmit the virus to mammals such as pigs and humans (Alexander, 2000; Webster et al., 1992), hampering the control of viral mutants. Moreover, AIV H9N2-infected chickens are vulnerable to secondary infection by pathogenic microbes, which may consequently cause severe commercial loss.

Although immunization with vaccines is not complete, it is one of the most promising control measures for the LPAI H9N2 to date. Modified live virus (MLV) vaccines have been used in many countries to control AIV (Pavlova et al., 2009; Song et al., 2007; Steel et al., 2009), since vaccination with inactivated AIV is usually efficacious but requires individual administration and more time to develop protective immunity. However, the possibility of reassortment between vaccine viruses and field isolates, and of mutations from low-pathogenic to highly pathogenic viruses may create serious safety concerns about using MLV strains as poultry vaccines. Therefore, prior stimulation of the immune system using some immunomodulators followed by vaccination with inactivated vaccines may be needed to confer better protective immunity within short time and may be promising in controlling LPAI H9N2.

The World Health Organization (WHO) has urged meat producers to use environmentally friendly alternative methods to control disease. Cytokines, as natural mediators of the immune response, are alternatives to conventional therapeutics. The efficacy of cytokine therapy has been demonstrated in several human and animal studies (Kim et al., 2010; Liu et al., 2010; Rhodes et al., 2011; Zuckermann et al., 1998). The utilization of chicken cytokines is becoming more feasible with the recent cloning of a number of cytokine genes, since the chicken's immune system is similar to that of mammals. Chicken interferon-alpha (chIFN-α) belongs to type I IFNs and plays an essential role in the host antiviral response through the stimulation of T-dependent lymphocyte system and induction of numerous IFN-stimulated genes (ISGs) (Li et al., 2005; Rose, 1979; Sekellick et al., 1994). There is evidence that chIFN-α administered by oral ingestion or intravenous injection inhibits many epidemic avian viruses, such as infectious bronchitis virus (IBV) (Pei et al., 2001), infectious bursal disease virus (IBDV) (O'Neill et al., 2010), Newcastle disease virus (NDV) (Wei et al., 2006), and AIV (Meng et al., 2011; Wei et al., 2006). Recently, it was reported that the oral administration of a recombinant chIFN-α protein can protect specific pathogen-free (SPF) chickens from AIV H9N2 challenge (Meng et al., 2011), which provides a new option in the prevention and therapy of AIV H9N2 infection. However, the mass administration of chicken cytokines to control poultry diseases is limited by cost, labor, time, and protein instability. Therefore, it is necessary to develop an effective delivery system for the mass administration of chicken cytokines to overcome these limitations.

To this end, our previous study reported that an attenuated aspartate β-semialdehyde dehydrogenase (Asd)-negative *Salmonella enterica* serovar Typhimurium strain devoid of antibiotic resistance genes could be an effective delivery system for the mass administration of cytokines without the need for antibiotic selection (Kim et al., 2010). Furthermore, cytokines produced by *S. enterica* serovar Typhimurium may be able to provide immunomodulatory functions to both mucosal and systemic sites, because orally administered *S. enterica* serovar Typhimurium can colonize the secondary lymphoid and nonlymphoid tissues including the lymph node, spleen, and liver, as well as the gut-associated lymphoid tissues (Peyer's patch) (Medina and Guzmán, 2001). The specific aims of the present study were to provide insight into the values of attenuated *S. enterica* serovar Typhimurium as a carrier for chIFN-α protein to control LPAI H9N2 in chickens. Oral administration of single dose of *S. enterica* serovar Typhimurium expressing chIFN-α alleviated clinical signs caused by respiratory infection with AIV H9N2 and reduced the excretion of AIV H9N2. Furthermore, specific immune responses against AIV H9N2 challenge in chickens orally administered *S. enterica* serovar Typhimurium expressing chIFN-α were evaluated.

2. Materials and methods

2.1. Animals and ethics statement

SPF leghorn layer (white) chickens were obtained from Jinan Baizhun Biologic Inspection, China, and were reared with formulated commercial feed and water provided *ad libitum* throughout the whole experimental period. All experimental procedures and animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committees of Chonbuk National University. The animal facility of the Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

2.2 Cells and viruses

Low pathogenic avian influenza A virus (AIV) H9N2 strain, A/Chicken/Korea/01310/2001 (01310), was a kind gift from the National Veterinary Research and Quarantine Service of the Republic of Korea (Choi et al., 2008). AIV H9N2 (01310) was propagated by inoculating in the allantoic cavity of 10-day-old embryonated eggs and allantoic fluid was harvested 96 h after inoculation. Virus in the allantoic fluid was titrated using a standard hemagglutination test (Hirst, 1942) and the infectious viral titer was determined by using 10-day-old embryonated eggs, as previously described (Swayne et al., 1998).

2.3 Bacterial strains, plasmid, media, and growth conditions

Escherichia coli χ6212 ($F^-\lambda^-\phi80\Delta$ (*lacZYA-argF) endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1* Δ*asdA4*) (Nakayama et al., 1988) was used as the host strain for construction of the Asd+ vectors. Attenuated *S. enterica* serovar Typhimurium χ8501 (*hisG* Δ*crp-28* Δ*asdA16*), which was kindly provided by Dr. H.Y. Kang (Pusan National University, Korea) (Kang et al., 2002), was used for the delivery of swIFN-α proteins. pYA3560 Asd+ plasmid was derived from pYA3493 Asd+ plasmid by changing pBR *ori* gene (origin of replication of pBR322 plasmid) with