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Generation and characterization of new monoclonal antibodies against swine origin 2009 influenza A (H1N1) virus and evaluation of their prophylactic and therapeutic efficacy in a mouse model

Sheng-Fan Wang [a, b, *], Sung-Pin Tseng [a], El-Wui Loh [c], Wen-Hung Wang [d], Ming-Chun Li [e], Kuan-Hsuan Chen [b], Wan-Chi Tsai [a], Yuan-Ming Lee [f], Huan-Yuan Chen [g, h], Fu-Tong Liu [g, h], Yi-Ming Arthur Chen [b, i, **], Jason C. Huang [j, k, ***]

[a] Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[b] Center for Infectious Disease and Cancer Research, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[c] Center for Evidence-based Health Care, Taipei Medical University, Shuang Ho Hospital, New Taipei City 23561, Taiwan
[d] Department of Medicine, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[e] Department of Pediatrics, Taipei City Hospital, Yang-Ming Branch, Taipei 11146, Taiwan
[f] Department of Laboratory Medicine, National Yang-Ming University Hospital, Yi-Lan 260, Taiwan
[g] Institute of Biomedical Sciences, Academia Sinica, Taipei 11578, Taiwan
[h] Department of Dermatology, University of California at Davis, CA 95816, USA
[i] Department of Microbiology, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[j] Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei 11272, Taiwan
[k] AIDS Prevention and Research Center, National Yang-Ming University, Taipei 11272, Taiwan

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ABSTRACT

In 2009, a swine-origin influenza A virus – A(H1N1)pdm09 – emerged and has became a pandemic strain circulating worldwide. The hemagglutinin (HA) of influenza virus is a potential target for the development of anti-viral therapeutic agents. Here, we generated mAbs by immunization of baculovirus-insect expressing trimeric recombinant HA of the A(H1N1)pdm09 strain. Results indicated that the mAbs recognized two novel neutralizing and protective epitopes-"STAS" and "FRSK" which located near Cb and Ca1 antigenic regions respectively and were conserved in almost 2009–2016 influenza H1N1 stains. The mAb 12E11 demonstrated higher protective efficacy than mAb 8B10 in mice challenge assay. Both mAb pretreatments significantly reduced virus titers and pro-inflammatory cytokines in mice lung post-infection ($p < 0.01$), and showed prophylactic and therapeutic efficacies even 48 h postinfection ($p < 0.05$). Combination therapy using the mAbs with oseltamivir pre- and post-treatment showed synergistic therapeutic effect in mice model ($p < 0.01$). Further investigation for clinical application in humans is warranted.

1. Introduction

A(H1N1)pdm09, a swine-origin influenza A virus isolated in April 2009, has been identified as a new reassortant strain. This virus spread rapidly from human to human and resulted in the raise of global influenza pandemic alert (LaRussa, 2011; Rossman and Lamb, 2010). Recent reports indicate that existing vaccines become ineffective because of antigenic variations in this pandemic strain (Chambers et al., 2016). To overcome the drawbacks, several broad-range prophylactic and treatment strategies have been proposed, including the use of monoclonal antibodies (mAbs) which is capable of targeting neutralizing epitopes specific to a subtype or

* Corresponding author. Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung 80708, Taiwan.
** Corresponding author. Department of Microbiology, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan.
*** Corresponding author. Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, No. 155, Li-Nong St., Section 2, Taipei 11272, Taiwan.
E-mail addresses: wasf1234@kmu.edu.tw (S.-F. Wang), arthur@kmu.edu.tw (Y.-M. Arthur Chen), jchuang2@ym.edu.tw (J.C. Huang).

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broadly shared among different subtypes. Several animal models have revealed that administration of mAbs via intranasal inoculation or intraperitoneal injection prevents influenza infection and limits virus spread to other organs (Harris et al., 2013; Kennedy et al., 2013; Yasugi et al., 2013).

The envelope glycoprotein of influenza A virus is mainly composed of hemagglutinin (HA) and neuraminidase (NA) glycoproteins. HA is a spike form trimeric structure on viral envelope which interacts with sialoside receptors on susceptible cells then cause infection (Chen et al., 2011). HA contains a globular head which shows higher immunogenicity to induce protective antibodies (Feshchenko et al., 2012; Wang et al., 2009a). Previous reports indicated that glycosylation is required for proper folding of HA and important for elicitation neutralizing antibody (Braakman et al., 1991; Marquardt et al., 1993; Medina et al., 2013). HA contains five antigenic regions near or around the receptor binding site which are involved in antigenic drift of influenza A viruses (for H1N1 the antigenic sites are Ca1, Ca2, Cb, Sa, and Sb) (Lin et al., 2008; Wang et al., 2009b).

Presently, two main countermeasures, vaccines and antiviral drugs, are used to fight against influenza virus. Vaccination is the basis of infection control (Feshchenko et al., 2012). However, the protection afforded by vaccination varies, depending on the antigenic match between the circulating virus and the vaccine selected candidates as well as the age and health status of the recipients (Carrat and Flahault, 2007). Neuraminidase inhibitors, such as oseltamivir (Tamiflu) and zanamivir (Relenza), and matrix ion channel inhibitors (amantadine), have been widely used in the treatment of influenza infection (Chang et al., 2010). These antiviral drugs have limited efficacy in delayed administration after the onset of illness (Midoro-Horiuti and Goldblum, 2014) and their widespread use may result in the emergence of drug resistant viral strains. Improvement of protective or therapeutic efficacies is therefore the priority global public health duty against influenza infection (Huang et al., 2009). MAb has been reported to be the potential candidate for influenza immunotherapy. Our goal was to develop high efficient viral neutralizing antibodies for influenza disease control and treatment.

Many systems utilizing yeast, baculovirus/insects, and mammalian cells have been established to produce suitable glycosylated antigens to induce neutralizing antibodies against influenza A virus. The baculovirus based generating glycosylated trimeric influenza HA (Tri-rHA) is viewed as a suitable candidate for vaccine development owing to its similar structure with the native trimeric form of HA (Hu, 2005).

Here, we generated mAbs via immunization mice with the Tri-rHA of A(H1N1)pdm09, and characterized their properties. We found that the mAbs displayed neutralization to A(H1N1)pdm09 infection. The mAbs recognized two new conserved and protective epitopes which were not disclosed previously, and displayed prophylactic and therapeutic efficacies in mice model. Further, less neutrophil infiltrations and lower proinflammatory cytokines were detected in the lung of mAb treated mice inoculated with A(H1N1)pdm09 virus.

2. Material and methods

2.1. Ethics statement

All animal procedures performed in this study were in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Animal use protocols were evaluated and approved by the Institutional Animal Care and Use Committee of Academia Sinica, Taiwan (IACUC approval No. 10-11-090). The pandemic swine origin H1N1 influenza A virus has been identified as biosafety level-2 (BSL-2) pathogen. The *in vitro* virus culture and infectivity assay were conducted in BSL-2 laboratory. The influenza virus mice challenge assay was performed in the influenza animal center of Academia Sinica, where the assay was under biosafety level 3 (BSL-3) containment. All infections and sample collections were performed under sodium pentobarbital anesthesia (60–80 mg/kg) and all efforts were made to minimize animal suffering. Body temperature, weight and survival of the mice were measured and monitored every day for 14 days postinfection. When the mice showed over 25% of body weight loss, they were considered to have reached the experimental end point and were humanely euthanized following IACUC guidelines to ameliorate suffering.

2.2. Cells and viruses

Madin–Darby canine kidney (MDCK) cells were maintained in Minimum Essential Media (MEM)(Gibco^{R}, Cat. No. 51200038) supplemented with 10% Fetal bovine serum (FBS) and penicillin/streptomycin. Reagents for cell culture were purchased from Gibco Life Technologies. Swine origin A/California/7/2009(H1N1)-like strain (GenBank Accession No. KC781785) was offered by Taipei Veterans General Hospital. The subtypes were determined using real-time PCR and the antigenic characteristics were determined using hemagglutination inhibition (HI) and microneutralization (MN) assays. The virus titers were determined in 96-well microplates infected with approximately 50 cell culture infectious doses (Median tissue culture infectious dose; $TCID_{50}$) of virus by quantifying virus-induced cytopathic effect (CPE).

2.3. Recombinant A(H1N1)pdm09 trimeric HA protein construction and their application in generation of monoclonal and polyclonal antibody

We used the Bac-to-Bac® Baculovirus Expression System (Invitrogen, Cat. No. 10359-016) to generate the rHA proteins. The protocol for generating the trimeric protein was referenced to previous publication (Lin et al., 2011) and listed in the supplementary data. For mAb or pAb generation, the BALB/c mice were immunized intraperitoneal with 5 μg of rHA1 in 0.1 ml of PBS which was emulsified with an equal volume of complete Freund's adjuvant (Sigma Aldrich, Cat. No. F5881). Twice intraperitoneal booster of the same dose of rHA1 emulsified with incomplete Freund's adjuvant (Sigma Aldrich, Cat. No. F5506) was performed. A final boost was given 3 days before splenocytes were collected and fused to NS-1 myeloma cells, as described previously (Wang et al., 2009a). Clones producing specific individual antibodies were selected via dilution method (Wang et al., 2009a). Polyclonal antibodies against the rHA1 protein were prepared as follows: New Zealand white rabbit was immunized and boosted (two or three times total) with 100 μg rHA1 in 0.5 ml PBS emulsified with an equal volume of complete/incomplete Freund's adjuvant. Rabbit blood was drawn for assays after confirming the increase in antibody titers against the rHA1 and swine origin 2009 H1N1 influenza A virus. The immunized rabbit was our No.113 (R113) laboratorial immunization rabbit.

2.4. Hemagglutination (HA), immunofluorescent assay (IFA), hemagglutination inhibition (HI) and microneutralization assay (MN) assays

Detailed procedures of HA and IFA are described in previous publications (Lin et al., 2011; Wang et al., 2009a). The detail protocol was shown in supplementary data. Regarding HI and MN assays, both were performed according to WHO Manual on Animal