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Research paper

Preparation of monoclonal antibodies against poor immunogenic avian influenza virus proteins

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ABSTRACT

This study established a novel method of pre-screening peptides for monoclonal antibody (mAb) production. Whole virus particles were used as antigens to produce mAbs in the first stage. However, most mAbs obtained from this method were aimed toward hemagglutinin. For this reason, synthetic peptides were used as antigens for mAb production that aimed at the AIV proteins with low abundance or poor immunogenicity in the virus particle. The peptides that showed high immunogenicity were designed using bioinformatic tools for immunization. For high-throughput, a rabbit was used to screen the immunogenicities of the synthetic peptides. Those showed high immunity were used for mAb preparation in mice. Several new mAbs against PB2, PA, M1, M2, NS1 and NS2 proteins were successfully obtained in this study. Furthermore, the epitopes of M1 and NS1 mAbs were determined using competitive western blot assay and competitive ELISA. This study might simplify the mAb preparation and serves as the basis for developing mAb against poor immunogenic proteins.

1. Introduction

The avian influenza virus (AIV) is classified in the negative-stranded RNA virus in the *Orthomyxoviridae* family. Its proteome is comprised of 11 virus proteins, including two major surface proteins, hemagglutinin (HA) and neuraminidase (NA), the most abundant antigen to induce neutralizing antibodies and the other immune responses (Girard et al., 2005). Based on the higher immunogenicity and large amount in virus particles, neutralization mechanism by antibodies against HA or NA has been widely investigated. Various anti-HA monoclonal antibody (mAb) approaches have been major targets for influenza research (Ilyushina et al., 2004; Hoffmann et al., 2005; Stephenson et al., 2006; Veits et al., 2006). Besides HA and NA, the other virus components generally belonged to structural or functional proteins that are not located on the surface. These proteins with low abundance or poor immunogenicity in virus particles have been shown to play important roles in the virus replication cycle. Those proteins include M1, NS1, and polymerase proteins.

RNA segment 8 of AIV encodes two non-structural proteins, NS1 and NS2. NS1 is found abundant in AIV infected cells (Krug and Etkind, 1973) but not in virus particles (Lazarowitz et al., 1971; Krug and Etkind, 1973). It regulates several functions in the AIV life cycle. The main functions are associated with alterations in cellular and virus gene expression (Enami et al., 1994; Qian et al., 1994; Nemeroff et al., 1998), regulating apoptosis (Zhirnov et al., 2002) and protecting the influenza virus against the cellular cytokine response from the host (Hatada et al., 1999; Seo et al., 2002). NS1 is a suitable target for diagnostic assay applications. Therefore, several NS1 mAbs were generated via recombinant NS1 protein as an antigen to solve the low abundance problem (Tan et al., 2010).

The M1 protein is the most abundant virus protein that underlies the lipid envelope as well as not located on the virus surface (Garoff et al., 1998). It maintains membrane

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rigidity to sustain the virus structure and interacts with the cytoplasmic tails of the HA, NA, M2 proteins and ribonucleoproteins (Nayak et al., 2009). The M1 protein belongs to the poor immunogenic protein family. Only a few mAbs against the M1 protein were screened using this traditional method (Linke et al., 2011).

mAb is a powerful tool for virus research because it is a specific probe (Wiley et al., 1981; Bizebard et al., 1995; Fleury et al., 1998). Neutralizing mAb directly inhibits receptors and the active sites (Varghese et al., 1983; Webster et al., 1988; Fleury et al., 1998). The neutralizing effect mechanisms of anti-HA or anti-NA antibodies on virus infectivity have been widely investigated (Murphy et al., 1972; Barbey-Martin et al., 2002; Knossow et al., 2002). However, other virus components that belong to structural or functional proteins have been seen little progress in mAb preparation.

Most current protocols for producing mAb against AIV were used to either immunize virus particles or expression protein as an antigen (Oxford et al., 1981; Okuno et al., 1993; He et al., 2007; Yang et al., 2008; Prabakaran et al., 2009; Tan et al., 2010). However, it is difficult to induce poor antigens into an effective immune response. Synthetic peptides have the advantage of overcoming the aforementioned shortcomings because antigens focused on a specific fragment that increases immune response and the epitope recognized by the antibody is defined in advance (Lerner et al., 1981; Niman et al., 1983). Although this method has been widely used for mAb production, there has been relatively little research on AIVs.

This study used a new high-throughput strategy for the hybridoma technique to screen mAbs against the AIV proteins with low abundance or poor immunogenicity. This strategy might simplify mAb preparation and could serve as the basis for a new AIV pathogenesis study.

2. Materials and methods

2.1. Viruses

Influenza virus, A/chicken/Taiwan/2838V/00 (H6N1, 2838V) was grown in the allantoic cavity of 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs and purified by centrifugation with gradient sucrose. The purified virus was resuspended in TEN buffer (10 mM Tris-base, 1 mM EDTA, and 100 mM NaCl, pH 8.0) and inactivated with 1 mM binary ethyleneimine (BEI) at 37 °C for 24 hr before use.

2.2. mAbs preparation by virus particles

2838V was used as the antigen to immunize mice as previously described (Chen et al., 2010). The mAbs were generated from immunized 6-week-old BALB/c mice that were injected intraperitoneally with 100 μg of purified virus particles with complete Freund's adjuvant. The mice were boosted with 50 μg of virus particles in incomplete Freund's adjuvant every two weeks for a total five injections until the antibody titer was detected by western blot assay. At the last boost, the mice were injected with 50 μg of virus particles without any adjuvant. Five days later spleen cells fused with myeloma cells (SP2/0-Ag14) were harvested from the immunized mice. The hybridoma cells were selected using hypoxanthine–aminopterin–thymidine. These cells with apparent titers were screened by western blot assay as described below with 2838V purified virus separated using 15% SDS-PAGE, obtaining single clones for mAbs using limiting dilution.

2.3. Synthetic peptides for immunization

The experimental design for mAb preparation is summarized in Fig. 1. It consists of the peptides design, rabbit immunization and mouse mAbs preparation. The antigenicity of PB2, PB1, PA, NP, M1, M2, NS1 and NS2 proteins for the peptides design were predicted using Lasergene-Protean (DNASTAR, Madison, WI). Besides the Jameson and Wolf method (Jameson and Wolf, 1988) in that program, the selection rules included the following: (1) the peptides contained both hydrophobic and hydrophilic residues that preferably lay in long loops or beta-sheets in the secondary structure; (2) the peptides location had to avoid helical regions, N-glycosylation sites and phosphorylation sites. The NetNGlyc 1.0 and NetPhos 2.0 servers were used to predict potential N-glycosylation sites and phosphorylation sites (Blom et al., 2004).

Three peptide fragments in each protein were designed from 2838V AIV. Each peptide was 20 amino acids in length (Table 1). These peptides were synthesized using high-density multiple antigenic peptide systems that attached through the C-terminal to a core matrix of eight-branching lysine by Genomics BioSci &Tech Co., Ltd. (Taipei, Taiwan). Each peptide was purified using high performance liquid chromatography and verified using mass spectrometry.

2.4. Rabbit immunization with synthetic peptides

The immunogenicity of synthetic peptides was tested in rabbits before mAb preparation in mice. Three rabbits were used at the beginning. Since all three showed the same result after immunization by western blot with virus particles, one representative rabbit was used for the following experiment for comparison. To check the peptide antigenicity we mixed all of the 24 peptides as antigens to inject a rabbit intraperitoneally with 100 μg each of the peptides with complete Freund's adjuvant. In the following injections, the rabbit was boosted with half of the peptides in incomplete Freund's adjuvant every two weeks for a total seven injections until antibody titers were detected. At the same time, blood was collected from the auricular artery for enzyme-linked immunosorbent assay (ELISA) and competitive ELISA as described below to monitor the antiserum against synthetic peptides. The use of rabbit and mice in this research was approved by the Institutional Animal Care and Use Committee, National Taiwan University.

2.5. mAb preparation with synthetic peptides

The mAbs against the 2838V virus proteins with low abundance or poor immunogenicity were generated from immunization with the 6-week-old BALB/c mice using the synthetic peptides following the new strategy (Fig. 1). Three peptides from each protein were mixed to immunize mice for mAb preparation by the aforementioned methods. The hybridoma cells were screened by ELISA with synthetic peptides. In addition, the M1 and NS1 were then screened