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Original Article

Characterization of Nucleoprotein Extracted from Human Influenza A Virus Cultured in Two Different Cell Lines

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Abstract

Background and Aims: Influenza virus nucleoprotein (NP) has the capacity to be used as subunit vaccine, but little is known about the impact of different cultures on its structure. In the present study we aimed to evaluate and compare the Isoelectric focusing (IEF) property of extracted viral nucleoproteins derived from Madin Darby canine kidney (MDCK) cell line and embryonated chicken eggs (ECE).

Materials and Methods: Influenza virus strain A/NewCaledonia/20/99/H1N1 was propagated and grown in allantoic sac of 10-11 day-old embryonated chicken eggs, and mammalian cell culture (MDCK) in parallel. Ribonucleoprotein extraction was conducted from two separate cultures and evaluated using isoelectric focusing gel strips.

Results: The results showed higher isoelectric pH in extracted nucleoproteins from MDCK as compared to embryonated chicken eggs.

Conclusion: It is possible that some amino acids have been replaced. Suggesting that the changing net charge of protein may be affect the conserved regions of the protein. Therefore, this could impact the new generation of vaccines construction based on conserved proteins.

Keywords: Nucleoprotein; MDCK; Embryonated chicken egg; Isoelectric focusing

Introduction

Influenza A virus is enveloped with segmented single-stranded, negative-sense RNA. Due to antigenic shifts and drifts in hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, high rate of morbidity and mortality occurs annually worldwide (1-6). This virus produces ten proteins from eight RNA segments (1, 7) from which. -HA, NA and M2- are inserted into the lipid envelope. The viral RNA is in conjunction with nucleoprotein (NP) and three polymerase proteins (PA, PB1, PB2) forming Ribonucleoprotein (RNP) complex (1, 5). Influenza virus NP is one of the most abundant and conserved proteins in the viral structure [1, 2]. The most important role of NP is exposing the basic nucleotides for efficient reading by the polymerase enzyme for replication and transcription (1, 8).

Vaccination is the most effective way of protection against most pathogens including influenza virus. Although vaccines are constructed based on

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circulating viruses, there is still long way to yield promising vaccines against seasonal flu outbreaks (6). Today, embryonated chicken eggs are one of the most commonly and reliable tools which are used to propagate the influenza virus for vaccine production (9). However, egg-allergic individuals are the main obstacles in this way (10). Alternatively, cell culture-based vaccine could alleviate this problem. Production of vaccine in cell culture allows for greater control of infection parameters than egg-based production (32). However, it is necessary to find the answer for this question that what would be the effect of different host systems on influenza internal proteins.

In the present study, we assessed influenza virus NP isoelectric (pI) properties in different host systems; MDCK cell culture & embryonated chicken eggs (CE).

Methods

Viruses

Influenza virus A/New Caledonia/20/99 (H1N1) was obtained from National Institute for Biological Standards and Control (NIBSC). The virus was propagated in both allantoic sac of 10- 11 day-old embryonated chicken eggs and a mammalian cell culture, MDCK. The virus was purified and concentrated as described previously (11) and stored in small aliquots at -70°C.

RNP Extraction

Isolation of RNP was conducted as described before (33) with partial modification. Briefly, stored virus was thawed at 37˚C, transferred to sterile thick-walled polycarbonate Beckman tubes and diluted with TES buffer (2 mM TES, 2 mM L-histidine, 100 mM NaCl [pH 7.4]). It was subjected to ultracentrifugation (55000 rpm or 18800 g 5°C 50 min) (Beckman-Coulter OptimaTM XL-100K) in a Beckman Ti90 rotor. Some of the supernatant and pellet was removed for HA assay and SDS-PAGE analysis. For the rest, the supernatant was removed and the pellet was resuspended by vortexing to a concentration of 2 mg viral protein per ml of extraction buffer (2 M NaCl, 0.02 M Sodium phosphate buffer [pH 7.4]). The nonionic detergent β-D-octyl-glucopyranosid (Sigma, USA) was then added in concentration of 2% (w/v) to solubilize the lipid bilayer as well as glycoprotins of the viral envelope. The suspension was mixed and placed in 37°C water bath for 45 min. Nucleocapsids were pelleted using ultracentrifugation as described in previous step. The resulting pellet and supernatant was taken out and stored at -20°C.

Hemagglutination assay

Hemagglutination (HA) was quantified as described by Mahy and Kangro (34), which is based on the influenza virus ability to aggregate red blood cells. Serial double dilutions of the test sample in duplicate were made in round-bottom 96-well microplates. HA units were calculated as the reciprocal of the highest dilution giving complete agglutination. Chicken red blood cells were used at a concentration of 0.5%.

SDS PAGE and Western blotting

Protein contents of various samples, virus and extracted viral proteins were quantified using modified Lowry method (12). Extracted proteins were loaded on 10% polyacrylamide gel containing SDS with recommended protocol by Laemeli in 1970 (13). The gel was stained using coomassie blue G250 (14,15). A marker polypeptide kit was applied including β-galactosidase (116 KD), Bovine Serum Albumin (66.1 KD), Ovalbumin (45 KD), Lactate dehydrogenase (35 KD), restriction endonuclease BSP 98 (25 KD), β-lactoglobuline (18 KD) and Lysozyme (14 KD). The polypeptides were blotted against nitrocellulose membrane (Schleicher & schuell, Germany) using vertical electroblotting system. The membrane was washed with PBS and incubated overnight at 4°C in 5% skim milk (Merck, Germany) as blocking buffer. Following washing with PBS,

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membrane was incubated for 2 hr at room temperature in PBS containing 0.5% Tween20, and anti-NP monoclonal antibodies (US biological, USA) with shaking. After washing with wash buffer for a minimum of three times with gentle agitation for 5-10 min, membrane was exposed to horseradish peroxidase (HRP)-conjugated secondary anti-species antibodies (US Biological) for 1 hr at room temperature with shaking followed by adding TMB substrate for visualization of positive bands.

Isoelectric focusing

For isoelectric focusing (IEF) analysis, the Multiphor II Electrophoresis system with Immobiline DryStrip gels (IPG), pH 3-10 and length of 7 cm (Bio-Rad, California, USA) was used. The samples were solubilized in the rehydration solution containing 8 M urea, 4 % (w/v) CHAPS, 50 mM DTT, 0.2 % (v/v) Biolyte TM pH 3-10, 0.0002 % (w/v) bromophenol blue. The solution was applied to the reservoir slots of the Reswelling Tray for the IPG strips rehydration (overnight at room temperature). After that, the IPG strips were removed from the tray and positioned in the Immobiline DryStrip aligner for IEF. Table 1 shows the parameters used for isoelectric focusing of protein samples.

Results

Ribonucleoproteins from two different cultures were loaded on gel electrophoresis and the result is shown in figure 1. In order to show the NP protein, after purification and extraction of the virus, the usual procedure by SDS-PAGE and Western blot analysis was performed using monoclonal antibody against NP protein (Figure 2). Protein band of 56 KD indicated the presence of desired protein, and usage of anti-NP antibody in Western blot confirmed presence of the protein in the samples.

IEF strips with a limit of two for electro focusing PHI 3-10 (Bio-Rad) was used. As shown in figure 3, first bar shows the loaded proteins extracted from the culture of fertilized eggs and the second shows the loaded proteins extracted from the culture medium of MDCK. The results showed that the NP extracted from the culture of

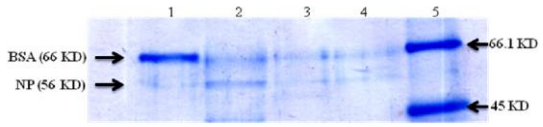

Fig. 1. Electrophoresis of the extracted proteins. Lane 1: BSA as a marker of 56 KD. lane 2: nucleoproteins extracted from the embryonated chicken egg. Lane 3 and 4: nucleoproteins extracted from MDCK. Lane 5: protein marker.

Fig. 2. Western blotting with monoclonal antibodies against the virus nucleoproteins. Lane 1: marker, lane 2: nucleoproteins extracted from MDCK. Lane 3: the extracted nucleoprotein from embryonated chicken egg.

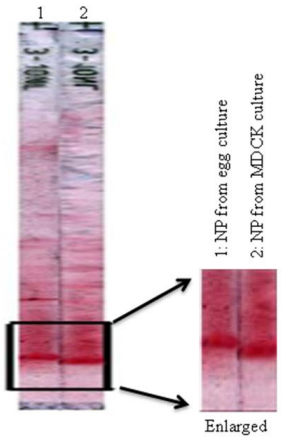

Fig. 3. The gel strip. First bar is NP extracted from the egg culture medium and the second is NP extracted from MDCK cell. Figure shows that NP from egg culture has higher pH than NP from MDCK medium culture.

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Table 1. Parameters used for IEF.

Phase	Voltage (V)	Duration H: min
1	250	00:20
2	4000	02:00
3	14000 v-h	03-04:00

fertilized eggs (first bar) owns higher pH than the MDCK (Second bar).

Discussion

The finding of this work highlights the alterations in isoelectric pH points in the NP extracted from two different cultures. It was shown that the MDCK-extracted NP owns higher pH than chicken egg-extracted NP.

The intrinsic genetic variability of influenza virus makes obstacles for vaccination programs, because the vaccines must be reformulated often and new vaccine must be designed based on current viral glycoproteins annually (16) which may cause devastating outcomes. To make a universal vaccine for influenza A virus, which includes the main seasonal flu strains and bird flu, as well as past pandemic strains, scientists are hoping to use conserved flu proteins that do not mutate much every year. One of the approaches to universal flu vaccine is using conserved internal proteins such as NP (3). Influenza A virus NP protein which is more conserved than HA is core antigen of virus. NP is bound to eight ssRNA genomes of influenza virus and protects the ssRNA against enzyme degradation (2). This protein with 498 amino acids in length is encoded by segment 5 in RNA. It is rich in arginine, glycine and serine residues. It has a net positive charge in neutral pH and an overall predicted pI of 9.3 (1). Several studies revealed that NP has the capacity to induce cell-mediated immune responses (17, 18). Therefore, every change into the primary structure of this protein could lead to changes of its expression especially if it occurs in the conserved regions of the protein causing evade from CTL response (19). There are also several evidence that the influenza virus components may be affected by the culture medium (20-23). It has been shown that, amino acid composition near the receptor binding pocket of HA alters based on the host, both in influenza A (20, 24) and B viruses (25, 26). One of the reasons to have different influenza A virus components in various host cells may be codon usage pattern of virus (27). The codon usage bias refers to differences in the frequency of occurrence of synonymous codons in coding DNA. Nucleoprotein is known to be a necessity for host specificity reactions (16). Therefore, its structure alteration could affect efficiency of constructed vaccines based on NP.

Literatures have reported that influenza virus HA N-glycosylation markedly depends on the host cell line used for virus production [28] and also it is shown that influenza virus propagated in bovine, human and chicken embryo cell cultures was maximally stable at low relative humidity (RH) in contrast to allantoic sac of chicken egg [29]. These results suggest that the internal proteins such as NP may be affected by different host systems as well. Although Shu *et al* showed that the NP of patient's viral samples in different places have no differences between MDCK and embryonated chicken egg (16), Hiromoto *et al* showed that influenza A/Hong Kong/156/97(H5N1) viruses cultivated in MDCK have higher pathogenicity than embryonated chicken egg viruses (20) and comparison of extracted internal proteins showed

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differences in the primary structure of proteins (20).

In this study, we used standard virus A/New Caledonia/20/99 (H1N1) to compare the host effect on mobility of nucleoprotein in isoelectric focusing. Viruses were cultivated in MDCK cell culture and embryonated chicken egg in parallel. Figure 3 shows bands at the end of IPG strips related to influenza nucleoprotein pI of 9-10 (1). The NP extracted from MDCK virus culture showed higher pI than ECE virus culture. Our results suggest that at least one of the basic amino acids was substituted by another one. Therefore, it shows that the changing net charge of protein may affect the conserved regions. Suggesting that this could be also occurred by codon usage bias (30). Hence, this may affect construction of new generation of vaccines based on conserved proteins, and this finding is consistent with results of others (20-23). Egg-free production of influenza vaccines eliminates the risk of allergic reactions to egg antigen and cell-derived vaccine safety has been demonstrated previously (25, 31). The replacement of egg-based influenza vaccine manufacture by cell-culture-based manufacture in future seems inevitable, but the pace remains uncertain.

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