Biochemical and Biophysical Research Communications 403 (2010) 247–251

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Universal antibodies against the highly conserved influenza fusion peptide cross-neutralize several subtypes of influenza A virus

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ARTICLE INFO

Article history:
Received 1 November 2010
Available online 13 November 2010

Keywords:
Influenza virus
Universal antibodies
Fusion peptide
Cross-protection

ABSTRACT

The fusion peptide of influenza viral hemagglutinin plays a critical role in virus entry by facilitating membrane fusion between the virus and target cells. As the fusion peptide is the only universally conserved epitope in all influenza A and B viruses, it could be an attractive target for vaccine-induced immune responses. We previously reported that antibodies targeting the first 14 amino acids of the N-terminus of the fusion peptide could bind to virtually all influenza virus strains and quantify hemagglutinins in vaccines produced in embryonated eggs. Here we demonstrate that these universal antibodies bind to the viral hemagglutinins in native conformation presented in infected mammalian cell cultures and neutralize multiple subtypes of virus by inhibiting the pH-dependant fusion of viral and cellular membranes. These results suggest that this unique, highly-conserved linear sequence in viral hemagglutinin is exposed sufficiently to be attacked by the antibodies during the course of infection and merits further investigation because of potential importance in the protection against diverse strains of influenza viruses.

1. Introduction

Influenza viruses include the genera of A, B and C. Type A and B influenza viruses are generally associated with more severe disease. The frequent mutations of the influenza viral surface proteins, hemagglutinin (HA) and neuraminidase (NA), allow the virus to evade the host immune system, resulting in annual epidemics and occasional pandemics [1,2]. Currently available prophylactic and therapeutic strategies against influenza, including vaccines and antiviral drugs, have inherent drawbacks. Specifically, while vaccines offer the best protection by inducing neutralizing antibodies against HA and NA antigens, current vaccines only protect against specific influenza strains [3,4]. Moreover, strains resistant to currently approved antivirals have been increasingly isolated in patients receiving these drugs [4–7]. They include M2 ion channel blockers (amantidine and rimantidine), which block un-coating of incoming viruses, as well as neuraminidase inhibitors (oseltamivir and zanamivir), which inhibit viral release from infected cells [5,6]. It is therefore of interest to explore alternative approaches which target the conserved sequence of influenza virus.

The HA protein is an attractive target for preventive and/or therapeutic intervention because it plays critical roles in the early stages of virus infection by binding to the viral receptor (sialic acid) on the target cells and mediating the fusion of viral and cellular membranes [8]. Development of antibodies (Ab or Abs) with broad reactivity against diverse viral strains could be challenging as HA of influenza A has two groups and 16 subtypes (H1–H16) [9–11] and it varies amongst not only the different subtypes but also the many different strains from the same subtype [12]. Recently, we [13] and others [14] identified a family of cross-subtype broadly neutralizing antibodies (BnAbs) that bind to a conformational dependent pocket-like epitope in the stem region of HA. The epitope is highly conserved among all group 1 influenza A viruses (H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16) and is formed by two elements critical to the pH-induced conformational change, the "fusion peptide" and the αA-helix. As such, the BnAbs neutralize all group 1 influenza A viruses by inhibiting the release of the fusion peptide, the first step in membrane fusion [13,14]. However, the BnAbs do

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doi:10.1016/j.bbrc.2010.11.030

not neutralize group 2 influenza A viruses (H3, H4, H7, H10, H14 and H15), which appear to be structurally different from group 1 viruses [13,14]. Also of note is that the conformational epitope targeted by the BnAbs is not universally conserved in type B and C influenza viruses. Previously we have shown through comprehensive bioinformatics analyses that the 14 amino acids (a.a.) at the N-terminal of the fusion peptide are the most highly conserved sequence in type A and B influenza viruses [15]. We have also demonstrated that mono-specific Abs against this peptide are capable of quantitatively binding to virtually all viral HA proteins [15,16]. Herein, we report that these Abs can inhibit viral growth by primarily impeding the fusion process between the virus and the target cells.

2. Materials and methods

2.1. Cells, viruses and recombinant proteins

Madin-Darby canine kidney (MDCK) cells, HeLa cells and 293T cells were obtained from ATCC and maintained as previously described [13,17], as were the preparation and titration of the influenza A strains A/Puerto Rico/8/34 (H1N1) (hereafter referred to as PR8), A/New Caledonia/20/99 (H1N1) (hereafter referred to NC), and A/New York/55/01 (H3N2) (hereafter referred to as NY).

2.2. Universal antibodies against hemagglutinins

Mono-specific universal Abs generated in rabbits against the N-terminal 14-a.a. of fusion peptide have been previously described [15,16]. The Abs, designated as Uni-1, were purified by affinity columns using the peptide as binding ligand as described previously [15]. F10, the positive control used in this study, binds to a conformation-dependent pocket-like epitope in the stem region of HA [13].

2.3. Immunoprecipitation

MDCK cells were infected with virus at multiplicity of infection (MOI) of 0.01 for 24 h at 37 °C. Supernatant was then removed and cells were lysed. Five hundred micrograms of cell lysate proteins was then incubated with 2 μg of Abs for 2 h. The antigen–antibody complexes were then immunoprecipitated using Catch and Release Reversible Immunoprecipitation System (Millipore, Billerica, MA) according to the manufacturer's instructions. Each experiment was conducted at least three times unless otherwise specified.

2.4. Mass spectrometric analysis

The immunoprecipitated proteins (antibody pull-down proteins) were processed as previously described [18]. Briefly, tube gel samples were sliced, the cysteines cleaved and capped, the proteins digested with trypsin and the peptides were then extracted. Nanoflow LC MS/MS analyses was performed using a Nano-Acquity ultra-performance liquid chromatography system (Waters, Mississauga, ON) with a BEH130 C18 analytical column coupled to a LTQ-FT Ultra ICR mass spectrometer (Thermo Fisher, Ottawa, ON). Mass spectral data were acquired in the data-dependent mode: ion-trap MS/MS at a normalized collision energy of 35% mode following a full FT-MS at a resolution of 100,000 at m/z 440.

2.5. Protein identification

The mass spectrometric data were searched against the National Center for Biotechnology Information (NCBInr) database using Mascot Server (Matrix Science, Boston, MA) specifying tryptic digestion and up to 2 missed cleavage sites, fixed modifications of carbamidomethylation of cysteines, variable modifications of deamidation of asparagine and glutamine, and methionine oxidation. Mass tolerances were set to 10 ppm for the FT MS peaks and 1 Da for ion-trap MS/MS fragment ions.

2.6. Plaque reduction assay

Plaque reduction assays were performed as described previously [17]. In brief, 100 plaque forming units (pfu) of virus were incubated alone or with 100 μg/ml Uni-1 Abs or control rabbit IgG Abs at 37 °C for 30 min. The virus-Ab mixture was then transferred onto confluent MDCK cell monolayers in 6-well plates and incubated at 37 °C for 1 h. The inoculum was removed and the cells were then washed and overlaid with agar. After 3 days of incubation at 37 °C, the overlay was discarded and the plaques were visualized by staining with 0.1% crystal violet.

2.7. Microneutralization assay

Microneutralization assays were carried out as described previously [13] with slight modifications. Briefly, 100 median tissue culture infectious doses ($TCID_{50}$) of viruses were mixed with an equal volume of log2 serial dilutions of Ab stock solution (200 μg/ml) in 96-well plates and incubated for 1 h at 37 °C. Virus-Ab mixture was transferred onto confluent monolayers of MDCK cells and incubated at 37 °C for 20 h. Cell monolayers were washed twice with PBS, fixed with ice-cold 80% acetone for 10 min and nucleoprotein (NP) viral antigen detected by indirect ELISA [13]. Results represent mean ± SD from three replicates.

2.8. Neutralization of H5N1-pseudotyped virus

The plasmids for the single-round HIV luciferase reporter virus pseudotyped with viral envelopes of H5-TH04 were described previously [13]. H5N1 pseudovirus was generated by co-transfection of 293T cells with 4 plasmids: HA-expressing plasmid, HIV packaging vector pCMVΔR 8.2 encoding HIV-1 Gag-Pol; transfer vector pHIV-Luc encoding the firefly luciferase reporter gene under control of the HIV-1 LTR; and N1-expressing plasmid pcDNA3.1-N1. Viral supernatants were harvested at 36 h post-transfection and stored at 4 °C until used. The neutralization assay was performed as previously described [13].

2.9. Cell fusion inhibition assay

Cell fusion inhibition assays were conducted as described previously [13]. Approximately 90% confluent HeLa cells in six-well plates were transfected with pcDNA3.1-H5-TH04 plasmid (3 μg total DNA per well) using lipofectamine 2000 (Invitrogen, Burlington, ON) according to manufacturer's instructions. After ~30 h of transfection, the culture medium was replaced with 1 ml of medium containing Uni-1 or control Abs at 100 μg/ml or 10 μg/ml for 2–3 h at 37 °C. The cells were washed twice with PBS and incubated with low-pH fusion buffer (150 mM NaCl plus 10 mM Hepes, adjusted to pH 5.0) for 4–5 min. They were then returned to the standard culture medium for 2–3 h at 37 °C, finally fixed with 0.25% (v/v) glutaraldehyde and stained with 0.1% crystal violet. Photomicrographs were taken at 10× magnification.

3. Results

3.1. Binding of Uni-1 to HA protein in virus-infected cultures

The mono-specific Abs, designated as Uni-1, were raised against the only universally conserved sequence in fusion peptide (the

amino terminal 1–14 amino acid of the HA2 subunit) of all influenza viral HA proteins [15]. Uni-1 showed remarkable specificity as no cross-reactivity to the proteins derived from allantoic fluids could be detected in Western blot [15]. Even though it was known that Uni-1 was able to quantify HA proteins in vaccines and recombinant HA [15,16], it was unclear whether Uni-1 could bind to native HA proteins from infected cell cultures. Here, the ability of Uni-1 to bind native HA associated with other viral proteins in virus-infected cultures was determined. To this end, extracts from cells infected with PR8 virus were immunoprecipitated using Uni-1 Abs, F10 [from Ref. 13] as a positive control and normal IgG as negative control. The immunoprecipitates were then subjected to MS/MS analyses. As shown in Table 1, LTQ FT LC MS/MS analyses revealed that Uni-1 and the positive control (F10) were able to precipitate HA associated with other major viral proteins in virus-infected cell cultures, while no viral protein was found to be precipitated with the IgG control, indicating that Uni-1 was able to bind to HA protein from infected cultures.

3.2. Inhibition of virus replication by Uni-1 Abs

Having observed that Uni-1 could bind to HA proteins of infected cell cultures, we next investigated whether it could inhibit virus replication in cell cultures. To this end, Uni-1 Abs were first tested by plaque reduction assay using the PR8 virus. As shown in Fig. 1, pre-incubation of Uni-1 Abs with PR8 virus for 30 min resulted in remarkable reduction of plaque size compared to IgG control. This finding prompted us to determine whether Uni-1 could neutralize diverse strains of viruses. As shown in Fig. 2, pre-incubation of Uni-1 with H1N1 (PR8), H1N1 (NC) and H3N2 (NY) viruses resulted in a substantial reduction of viral replications (panels A–C) with similar observation of inhibition of pseudotyped H5N1 viruses (Fig. 2, panel D). These results suggest that Uni-1 can inhibit replication of diverse strains of virus in a concentration-dependent fashion.

3.3. Uni-1 Abs inhibit viral fusion with the cells

To elucidate the mechanisms underlying the inhibition of virus replication by Uni-1 Abs, we investigated whether Uni-1 inhibited the fusion step following the initial binding of the virus to cells. Like F10 [see Ref. 13], Uni-1 failed to prevent binding of the virus to the cells (Fig. S1, Supplementary materials). On the other hand, significant inhibition of cell fusion was observed with Uni-1 Ab treatments (Fig. 3 panels E and F). As expected, the positive control (F10) prevented cell fusion (Fig. 3 panels C and D) while massive cell fusion was observed if no antibody was added (Fig. 3, panel B). It is of note, however, that Uni-1 is less potent than F10 since at 10 μg/ml (panel F) it could not completely inhibit polykaryon formation while none was observed with F10 at the same concentration (Fig. 3, panel D). These results may suggest that the conformational epitope targeted by F10 might be better exposed to the surface to allow antibody binding than the linear epitope recognized by Uni-1.

4. Discussion

Current influenza vaccines provide substantial protection in humans [3,19–21]. However the neutralizing Abs they induce are mainly strain-specific [3,22]. Most of these Abs target the highly variable immuno-dominant regions of the receptor-binding domain of HA; therefore they cannot induce heterosubtypic immunity [3,13]. Although it has always been thought that heterosubtypic immunity is mainly mediated by cross-reactive cytotoxic T lymphocytes (CTLs) [23,24], which target conserved epitopes in the viral internal proteins such as nucleoprotein and matrix protein

Table 1
Identification of viral proteins by mass spectrometric analysis.

Antibody	MDCK cells	Detection of influenza H1N1 proteins Number of peptides and protein sequence coverage (%)			
		HA	M1	NS1	NP
Uni-1	H1N1 infected	2 (3%)	6 (35%)	6 (43%)	2 (4%)
Uni-1	Uninfected	–	–	–	–
F10	H1N1 infected	2 (3%)	5 (29%)	4 (31%)	2 (5%)
F10	Uninfected	–	–	–	–
IgG	H1N1 infected	–	–	–	–

Lysates from the uninfected or infected MDCK cells with influenza A PR8 were immunoprecipitated by the specific antibody Uni-1, with F10 as positive control and IgG as negative control. The proteins were then identified by mass spectrometry. Reproducible results were achieved by parallel analyses from two independent experiments. Only the specific antibody-binding proteins are listed, whereas several non-specific binding proteins (albumin, ribosomal protein, tubulin, vimentin, elongation factor, high mobility group, Hnrp, etc.) are not shown since those were also observed in the control samples. Dashes denote the undetected proteins in the pull-down extracts. The abbreviations used here are hemagglutinin precursor (HA, gi|66239966), matrix protein M1 (M1, gi|4996868), non structural protein 1 (NS1, gi|31096442), and nucleoprotein (NP, gi|187763982).

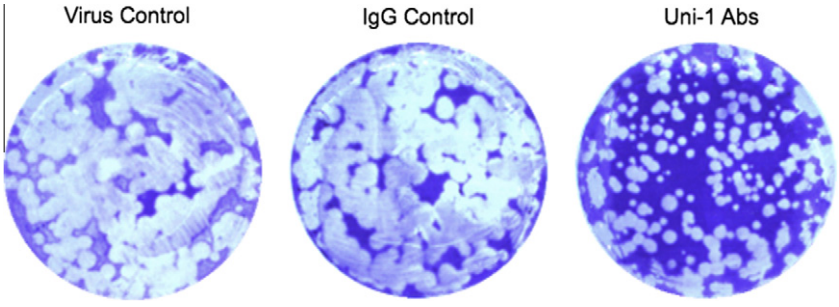

Fig. 1. Uni-1 Abs reduced the size of plaques. Influenza PR8 virus (100 pfu) was pre-incubated alone or with 100 μg/ml of Uni-1 Abs or control rabbit IgG for 30 min, then the virus-Ab mixture was transferred to confluent MDCK cells in a 6-well plate, incubated at 37 °C for 2 h and analyzed by plaque assay.

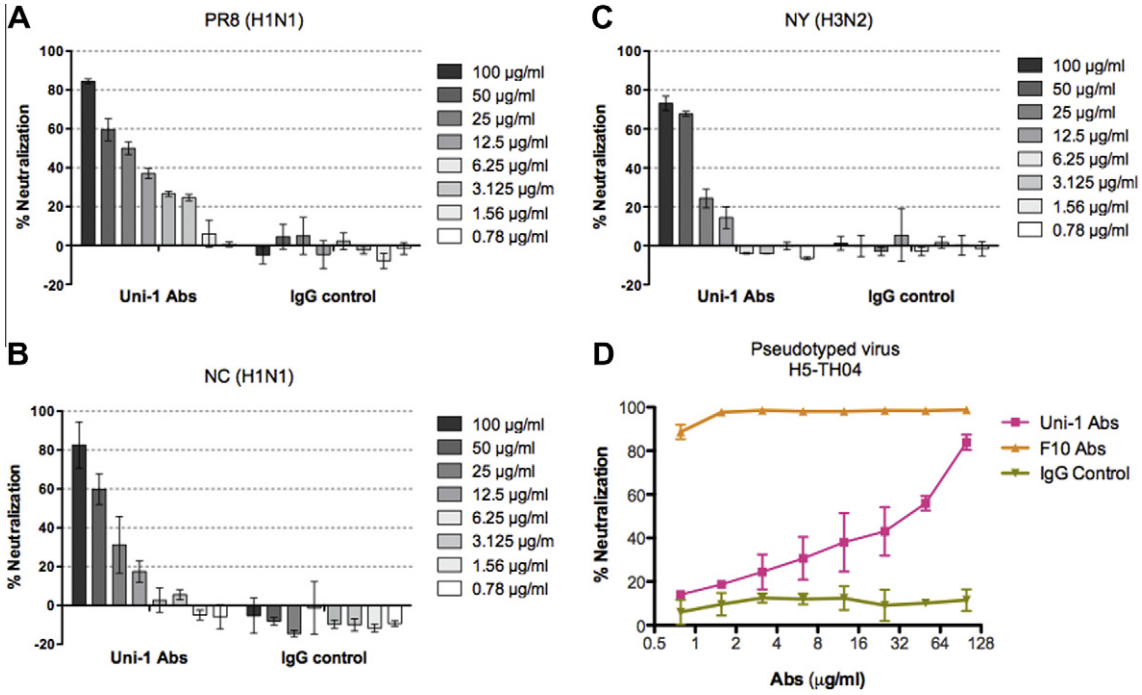

Fig. 2. Uni-1 Abs neutralize various influenza A subtypes. Uni-1 Abs or rabbit IgG control were tested for neutralization against the different viruses (A) influenza PR8 (H1N1), (B) influenza NC (H1N1) and (C) influenza NY (H3N2). (D) Neutralization of H5-TH04-pseudotyped viruses (virus-like particles with HIV-1 only cores expressing H5) by Uni-1 Abs and F10 Abs (used as positive control). Data are presented as percentage of neutralization with standard deviations. The experiments were conducted at least three times (same below).

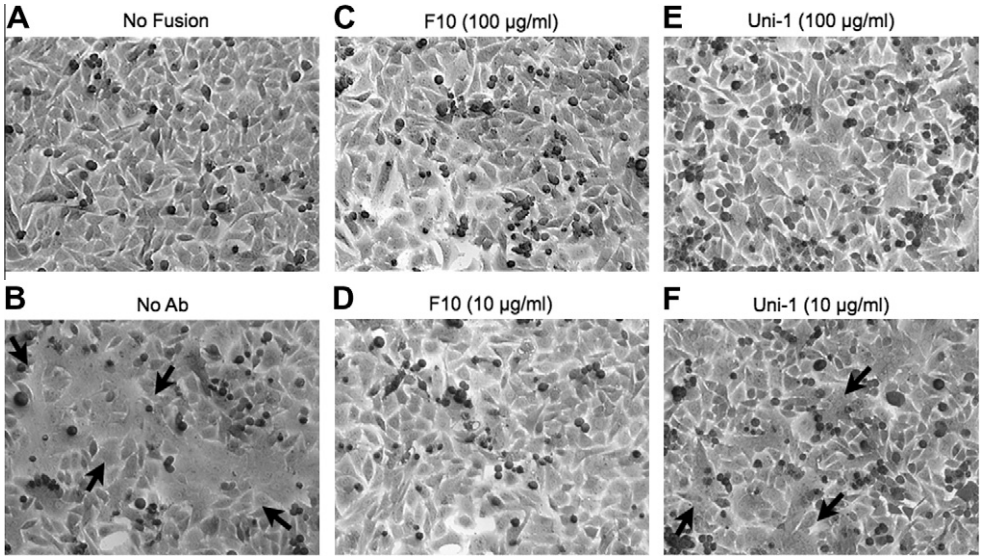

Fig. 3. Inhibition of cell fusion by Uni-1 Abs. HeLa cells were transfected with H5-TH04-expressing plasmid and exposed to a pH 5.0 buffer for 5 min in the presence or absence of Abs. (A) H5-Transfected HeLa cells without exposure to pH 5.0 buffer show no fusion. (B) H5-Transfected HeLa cells after exposure to pH 5.0 buffer in the absence of Abs show syncytia formation. (C) Syncytia formation was inhibited by F10 Abs (used as positive control) at a concentration of 100 µg/ml and (D) at 10 µg/ml. (E) Uni-1 Abs inhibited fusion at 100 µg/ml and (F) only showed partially inhibition at 10 µg/ml. Arrows indicate cell fusion. Pictures are representative of 10 fields.

[3,22,25], cross-reactive Abs against HA have also been long reported [26–29]; however, most of these Abs lacked neutralization activity. Recently, neutralizing Abs with broad reactivity against group 1 influenza A viral strains have been identified, targeting conformational epitopes comprised of a.a. from at least three distinct regions of the HA proteins, i.e., the HA1, HA2 and the fusion peptide at the N-terminus of HA2 subunit [13,14]. More recently, Wei et al. [30] reported that immunization with plasmid DNA encoding H1N1 influenza HA and boosting with seasonal vaccine or replication-defective adenovirus 5 vector encoding HA stimulated the production of broadly neutralizing influenza Abs in nonhuman primates. This suggested that induction of neutralizing Abs against the conserved stem region of HA in humans might also be achieved. Abs raised against the stalk of the HA proteins or HA2 domains have also been found to have broad neutralizing activities [31–33]. Through comprehensive bioinformatics analyses, we found that the N-terminal 14 a.a. of the fusion peptide in the HA2 subunit are the only universally conserved sequences among all influenza hemagglutinins [15]. Furthermore, an updated re-analysis of the current sequences revealed no sequence changes (Fig. S2, Supplementary materials).

More importantly, mono-specific Abs (denoted as Uni-1) against this linear epitope could quantitatively detect virtually all viral hemagglutinins [15,16].

In this communication, we found that Uni-1 could bind to HA proteins associated with other viral proteins and inhibit multiple strains of influenza virus in cell cultures. Moreover, an investigation of the mechanism has revealed that the inhibition of virus growth was due to Uni-1 impeding the fusion process. Clearly more studies are needed to determine whether Abs targeting the universally conserved epitope in viral hemagglutinins could protect animals from influenza in a suitable animal model, an issue which is not addressed in this study, given that Uni-1 is of rabbit origin. Nonetheless, the significance of our findings is clear: during the course of infection in tissue cultures, the viral fusion peptide is sufficiently exposed to allow access by neutralizing Abs, making it an attractive target for antiviral intervention and vaccine development, particularly because these Abs induced by the fusion peptide possess high specificity against all influenza viral hemagglutinins [15,16].

5. Competing interest statement

The authors declare no conflict of interest.

Acknowledgments

We thank Caroline Gravel for technical assistance and Monika Tocchi for editorial help. Drs. Richard Isbrucker and Shiv Prasad (Health Canada) are acknowledged for their critical review of the manuscript. AMH is supported by a Scholarship from King Abdulaziz University, through the Saudi Arabian Cultural Bureau in Canada.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.030.

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