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Monoclonal antibody recognizing SLLTEVET epitope of M2 protein potently inhibited the replication of influenza A viruses in MDCK cells

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ABSTRACT

The ectodomain of influenza A virus M2 protein (M2e) is composed of 24 amino acids and induces antibodies with inhibitory effect against a broad spectrum of influenza A subtypes in vitro and in vivo. Although relatively conserved, 21 M2e variants emerged in recent influenza A strains, most of the mutations appeared in the middle part of M2e domain. In this study, we characterized the in vitro inhibition efficacy of a monoclonal antibody (mAb) M2e8-7 recognizing the N terminus highly conserved epitope SLLTEVET (aa 2–9) which is common for both M1 and M2 proteins. Peptide binding assay showed that mAb M2e8-7 reacted strongly with M2e and 19 M2e variant peptides. The mAb M2e8-7 potently inhibited the replication of influenza A virus H1 and H3 subtypes in MDCK cells. Two important amino acids in M2e epitope, Threonine at position five and the Glutamic acid at position six, were identified to lead antibody-escaping variants. These results brought new insight in developing vaccine and therapeutic agents against influenza A virus infections.

Introduction

Influenza A is an enveloped negative single-stranded RNA virus that infects a wide range of avian and mammalian species and causes the greatest number of death worldwide. Current estimates are that 250,000–500,000 people die from influenza every year [1]. Hence, there is an urgent need to develop a new efficient vaccine against Influenza A. Although the virus expressed highly immunogenic proteins including hemagglutinin and neuraminidase at its membrane, these proteins are highly mutable and new epidemic strains arise every year. The high mutation rate, the ability of gene segments to reassort, and the huge pool of influenza viruses in birds and mammals explain their changing behavior and the difficulty in developing a permanent, long-lasting, and effective vaccine [2]. The development of an efficient vaccine against Influenza A remains the scientific and public health challenge.

The M2 protein consists of a 24-amino acid ectodomain (M2e) at its amino terminus, a single membrane spanning domain, and a 54-amino acid cytoplasmic tail. The M2 protein was though to conduct protons from low pH endosome to the virion interior. This activity is required both for viral replication and the proper maturation of some strains of influenza virus hemagglutinin [3,4]. The M2e protein is remarkably conserved among all influenza A viruses [5,6].

Efforts have been made to develop influenza A virus M2e protein as vaccine and therapeutic target. Antibodies induced by the M2e peptide displayed broad protection against influenza A viruses of both homologous and heterologous strains in vitro [7,8] and in vivo [9–11], which made M2e an attractive target for a 'universal' vaccine [12]. However, this vaccine requires multiple injections of high dose immunogens with one or two adjuvants to achieve high level neutralizing antibodies. This encumbers the application of M2e vaccine to humans, especially to young children, the elderly and immuno-compromised populations [13]. In a recent study, Liu et al. showed that the N terminus of M2e peptide, rather than the middle part and the C terminus, induced Abs with inhibitory effect [8]. In another study, Wang et al. compared the binding activity of mAbs recognizing either the middle part or the N terminus of M2e peptide. The results showed that the mAb targeting the N terminus displayed broader spectrum reactivity to the current M2e variants than mAbs targeting the middle part [13]. These data implied the therapeutic potential of the N terminus of M2e peptide to influenza virus infections.

In this study, we generated mAb against the N terminus highly conserved eight amino acids common for both M1 and M2 proteins (Fig. 1). This mAb exhibited high affinity to M2e peptide and cross

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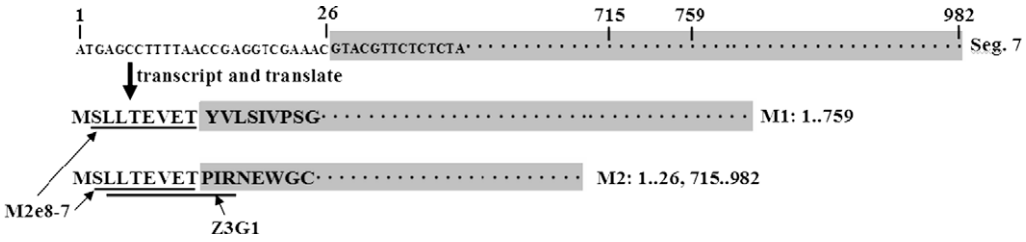

Fig. 1. Schematic presentation of influenza A virus M1 and M2 proteins coded by segment 7. The epitopes for mAb M2e8-7 and Z3G1 are underlined.

reacted with 19 of the current known 21 influenza A M2e variants. Two residues, the Threonine at position five and the Glutamic acid at position six, were identified critical for antibody binding. The replication of influenza A H1 and H3 subtypes in MDCK cells was efficiently inhibited by the mAb. However, the WSN virus with T5P or E6G mutations in the M proteins escaped the protection of this mAb. The mechanism of the inhibition and the potential of this mAb as diagnostic and therapeutic agent for influenza A virus infections were discussed.

Materials and methods

Cells and viruses. Madin-Darby canine kidney (MDCK) cell was maintained at 37 °C in a 5% CO2 incubator in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco), streptomycin (1000 μg/ml), penicillin (1000 U/ml) and 2 mM L-glutamine. Influenza A virus laboratory strains A/Shanghai/01/2008 (H1N1) and A/Shanghai/07/2008 (H3N2) were inoculated in MDCK cells in the presence of 0.25 μg/ml DPCK treated trypsin (Thermo).

Peptide Synthesize. The peptides M2e8-BSA (SLLTEVET conjugated with BSA at the C terminal), M2e8T5P-BSA (SLLPEVET conjugated with BSA at the C terminal), M2e8E6G-BSA (SLLTGVET conjugated with BSA at the C terminal), M2e7a-BSA (LLPEVET conjugated with BSA at the C terminal), M2e6a-BSA (LPEVET conjugated with BSA at the C terminal), M2e7b-BSA (SLLPEVE conjugated with BSA at the C terminal), M2e6b-BSA (SLLPEV conjugated with BSA at the C terminal), Loop-BSA (VIEKMNTQFTAVGKEFNNLEKRMENL conjugated with BSA at the C terminal), M2e and 21 M2e variants (M2eV1-M2eV21, Fig. 2) were synthesized with purity >85% at ShineGene Bio-Technologies, Inc. (Shanghai).

Immunization. Fifty microgram of synthesized M2e8-BSA was mixed with equal volume of complete Freund's adjuvant (Sigma) and used for immunization of each Balb/c mouse. Three weeks later, the mice were given two injections at 2-week interval with the same amount of protein mixed with equal volume of incomplete Freund's adjuvant. The serum was harvested 2 weeks after the last boost and titrated for each individual mouse.

Production of monoclonal antibodies. The specific anti-M2e8 mAbs were screened according to the standard hybridoma technique [14]. In brief, mouse myeloma cells (SP2/0) were fused with spleen cells of M2e8-BSA immunized Balb/c mouse. M2e8-BSA and Loop-BSA was coated with 0.1 M sodium carbonate buffer (pH 9.2) overnight at 4 °C. The specific M2e8-BSA positive and Loop-BSA negative mAb secreting hybridomas were screened by ELISA assay and then amplified. The isotype of the screened mAbs was determined using mouse monoclonal antibody isotype reagents (Sigma). The antibody concentrations of crude preparations were determined and complement proteins in ascitic fluids were inactivated at 56 °C for 30 min before use. The epitope mapping was performed with the N and C terminal truncated M2e8 peptides (M2e7a, M2e7b, M2e6a, and M2e6b).

Mutagenesis and reverse genetics. The reverse genetics system for generation of A/WSN/33(H1N1) viruses was kindly provided by Yoshihiro Kawaoka from University of Madison-Wisconsin. The plasmid pPolI-WSN-M for expressing WSN viral M RNA was mutated by using the site-directed mutagenesis kit (stratagene), resulting single substitution at L3F, L4P, T5P, E6G, E8D or E8K, respectively. Reconstruction of WSN virus and the 6 WSN mutants

	5 10 15 20	M2e8-7[a]	strains[b]
M2e	MSLLTEVETPIRNEWGCRCNDSSD	+++	nd
M2eV1	.....G...	+++	nd
M2eV2	.....E.K.....	+++	nd
M2eV3	.....E....G...	+++	nd
M2eV4	.....S.....G.	+++	nd
M2eV5	.....T....E...S....	+++	nd
M2eV6	.....T..G.E.....	+++	nd
M2eV7	.....T..G.....S....	+++	nd
M2eV8	.....T..G.E.K.....	+++	nd
M2eV9	.....LT..G.....S....	+++	nd
M2eV10	.....LTK.G.....S....	+++	nd
M2eV11	.....LT..G.E.K.S....	+++	nd
M2eV12	.....T..G.E.K.S....	+++	nd
M2eV13	.....KG.E.N.S....	+++	nd
M2eV14	.....T.DG.E.K.S....	+++	nd
M2eV15	.....HT..G.E.K.S....	+++	nd
M2eV16	..F.....E....G...	+++	nd
M2eV17	...P.....	+++	nd
M2eV18	.....D.LT..G.E...S....	+++	nd
M2eV19	.....K..T..G.E.K.S....	+++	nd
M2eV20	.....G...HT..G...K.S....	–	A/Nairobi/2041/2006(H3N2) A/chicken/HongKong/SF1/03(H9N2)
M2eV21	....P....HT..G.....S....	–	A/chicken/HongKong/YU427/03(H9N2) A/HongKong/1144/99(H3N2) A/Pigeon/HongKong/Y233/97(H9N2)

Fig. 2. Sequence alignment of M2e and 21 M2e variant peptides ([13]). The mAb M2e8-7 binding site is underlined. a: binding activity of mAb M2e8-7 to M2e and M2e variant peptides is evaluated in ELISA. '+++' represents positive binding at 10^8 mAb dilution, '–' represents negative binding at 10^2 mAb dilution. b: strains carrying the respective M2e sequence are indicated. 'nd' means investigation not did.

by using 12 plasmids was described by Neumann et al. [15]. The 6 WSN mutants contained single mutation in the first nine amino acids representing M2eV16–V21 (Fig. 2).

Binding activity. The binding assay was described elsewhere [13]. Briefly, M2e, the 21 M2e variants, M2e8T5P-BSA and M2e8E6G-BSA were coated with 0.1 M sodium carbonate buffer (pH 9.2) at 30 μg/ml. After blocking with PBS containing 1% BSA, 10-fold serial diluted anti-M2e8 mAb was added and incubated for 1 h incubation at 37 °C. After five times wash with PBST, peroxidase-conjugated goat anti-mouse immunoglobulins (Invitrogen) was added at dilution of 1:3000 and incubated for another 1 h at 37 °C. After five times wash, O-phenylenediaminde dihydrochloride peroxide solution (Sigma) was added and incubated for 20 min. The reaction was stopped by 3% H_2SO_4 and the absorbance was measured with microtitre plate reader at 450 nm (Thermo Scientific).

Inhibition assay. The inhibition assay was performed as described by Schmidtke et al. [16]. MDCK cells were seeded in the internal 60 wells of 96-well plate at a density of 3×10^4 cells/well in flat-bottom microtiter plate (Costar) and were incubated overnight at 37 °C with 5% CO2. MAb M2e8-7 was first diluted 20 times, and then serial twofold diluted in DMEM medium. One set of antibody dilutions was added to cells to detect the toxicity of the ascitic fluids. Fifty microliters diluted antibody was incubated with 50 50% tissue culture infective doses ($TCID_{50}$) of A/WSN/33(H1N1) or its mutants, A/Shanghai/01/2008(H1N1) or A/Shanghai/07/2008(H3N2) viruses in an equal volume of medium for 30 min at 37 °C. The mixture was then added to MDCK cells and inoculated for 1 h. The supernatant was then removed and 100 μl fresh medium containing 0.5% BSA and 0.25 μg/ml trypsin (for A/Shanghai/01/2008(H1N1) and A/Shanghai/07/2008(H3N2)) or without trypsin (for WSN and the 6 WSN mutants) was added to each well. Forty-eight hours later, the supernatant was removed and the cell monolayers were washed with PBS and stained with 50 μl staining buffer (0.2% crystal violet (w/v) in 20% ethanol (v/v)) for 30 min. After six times intensive wash, the OD at 630 nm of each well was quantified by spectrophotometer (Thermo Scientific). For each treatment, three parallel wells were prepared. All experiments were repeated two times independently. The protective rate was calculated as: protective rate = [(mean OD value of treatment wells − mean OD value of positive controls)/(mean OD value of negative control − mean OD value of positive controls)] × 100% [17].

Results

Generation of mAbs against influenza A virus M2e8 peptide

A previous study has demonstrated that epitopes in the N terminus of M2 protein could induce antibodies with inhibitory effect against both influenza A and B virus replications [8]. In this study, we compared the influenza A virus M1 and M2 proteins and found that the N terminus common nine amino acids of the two proteins were highly conserved (Figs. 1 and 2). Hence, we immunized mice with the eight common amino acids of M1 and M2 proteins (except the first methionine) conjugated with BSA (M2e8-BSA). After three times injection, the anti-serum titer reached 1:25,600 against M2e peptide, indicating that the M2e8-BSA protein was highly immunogenic. Eleven anti-M2e8 mAb secreting hybridomas (M2e8-1 to M2e8-11) were obtained. All these mAbs were IgG1 isotypes. One of them, M2e8-7, produced higher concentration of MAbs in supernatants and was used to produce ascitic fluids for the subsequent experiments. Further Elisa test to the BSA conjugated N and C terminal truncated M2e8 peptides (M2e7a-BSA, M2e7b-BSA, M2e6a-BSA and M2e6b-BSA) showed that the mAb M2e8-7 recognized the complete M2e8 SLLTEVET epitope (Fig. 1).

Binding assay against various M2e8 mutants

Statistic analysis revealed 21 M2e variants from all known influenza A strains [13]. Amongst them, six variants were found single mutation at mAb M2e8-7 binding site (Fig. 2: M2eV16-V21). The binding activity to these variants for mAb M2e8-7 was determined in ELISA. Results showed that mAb M2e8-7 at 10^8 dilutions strongly bound to M2e peptide and 19 synthesized M2e variants (Fig. 2). Two variants, M2eV20 in which mutations emerged at E6G, P10H, I11T, E14G and N20S, and M2eV21 which mutated at T5P, P10H, I11T, E14G, R18K, and N20S were not recognized by mAb M2e8-7 even at 10^2 dilutions. To identify whether single mutation in the mAb binding site or multiple mutations in the M2e peptide influenced the Ab-epitope interaction in M2eV20 and M2eV21, the binding activity of mAb M2e8-7 was further tested to two BSA conjugated M2e8 variants in which single mutation at T5P or E6G was introduced (M2e8T5P and M2e8E6G). The results showed that at dilution 10^2 mAb M2e8-7 could not react with both peptides, which was similar to the binding of M2eV20 and M2eV21, indicating single mutation T5P or E6G at mAb binding site was enough to abolish the interaction.

Inhibition of influenza A virus H1 and H3 subtypes

The inhibition activity of mAb M2e8-7 to A/Shanghai/01/2008 (H1N1) and A/Shanghai/07/2008 (H3N2) viruses was tested in MDCK cells. MAb at serial 2-fold dilution from 1:20 to 1:2560 was mixed with 50 $TCID_{50}$ viruses before infecting MDCK cells. As shown in Figs. 3 and 4, the replication of both viruses was inhibited by mAb M2e8-7 in a dose dependant manner. At the highest concentration 1:20, mAb M2e8-7 protected >90% cells from infection by both viruses. Fifty percent protection was observed between dilutions 1:160 to 1:320. No obvious inhibition effect was observed at dilution 1:2560. (Figs. 3 and 4).

Inhibition of WSN virus and 6 WSN mutants replication

Binding test has illustrated that single mutation in the mAb M2e8-7 binding site affect directly its binding efficacy. To access the influence of the six mutations in M2e8-7 binding site to the antibody inhibition efficacy, we constructed 6 WSN mutants by using reverse genetics technique, each variant was introduced a single mutation in the first nine amino acids of M gene, representing M2e8V16-21, respectively. Inhibition efficacy by mAb M2e8-7 from dilution 1:20 to 1:2560 against WSN virus and WSN mutants

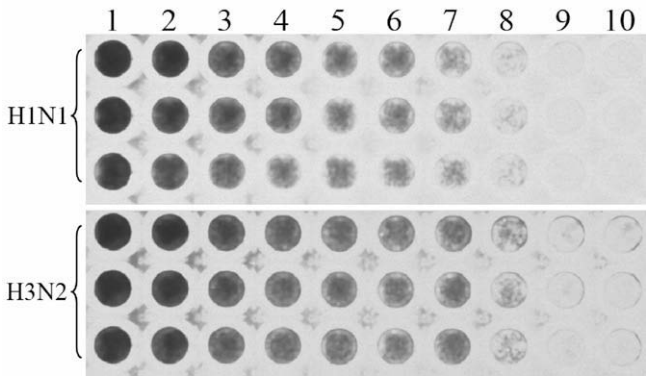

Fig. 3. Inhibition of the replication of A/Shanghai/01/2008 (H1N1) and A/Shanghai/07/2008 (H3N2) viruses in MDCK cells by mAb M2e8-7. 1, mock infection; 2–9, mAb M2e8-7 2-fold serial diluted from 1:20 to 1:2560 and inoculated with 50 $TCID_{50}$ viruses for 48 h. 10, infection with 50 $TCID_{50}$ viruses without mAb M2e8-7. Three parallel wells were prepared for all treatments.

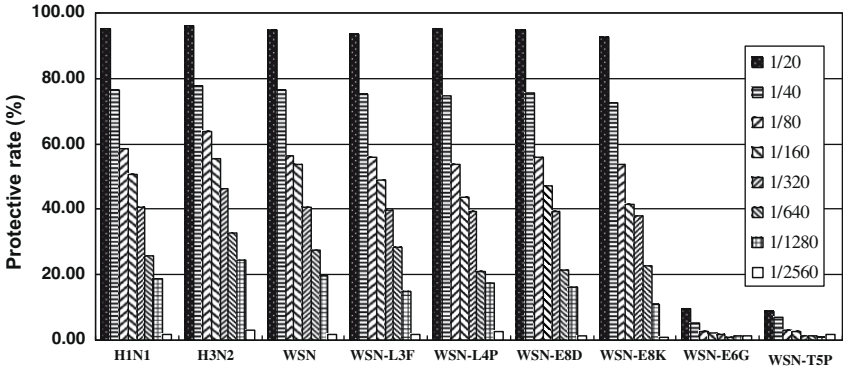

Fig. 4. Evaluation of the inhibitory activities of serial diluted mAb M2e8-7 to different influenza A viruses including A/Shanghai/01/2008 (H1N1), A/Shanghai/07/2008 (H3N2), A/WSN/33(H1N1) and 6 WSN mutants with single mutation at the N terminus of M proteins. The results represent mean value of three independent experiments.

with single mutation at L3F, L4P, E8D or E8K in the M proteins was similar or slightly diminished compare to influenza A H1 and H3 subtypes (Fig. 4). However, little inhibition effect was observed to the 2 WSN mutants with single mutation at T5P or E6G in the M proteins, even at the highest concentration 1:20 of mAb M2e8-7 (Fig. 4). These data confirmed that the Threonine at position five and the Glutamic acid at position six were important for the N terminal epitope of M2 protein.

Discussion

Current influenza vaccine strategy is mainly targeting the two major membrane glycoproteins, hemagglutinin and neuraminidase [18]. However, the high antigenic drift and occasional shift of the two proteins make vaccine production cumbersome and necessitate yearly revision of the vaccine seed strains [19]. Researches have been made to develop a universal vaccine with the highly conserved ectodomain 24 amino acids of M2 protein [8]. When conjugated to carrier proteins KLH or hepatitis B virus core (HBc), the M2e vaccine effectively conferred lethal influenza virus challenge in animal models [9,11,20,21]. Although the application of the M2e vaccine was currently encumbered by the necessity of high dose and multiple injections in humans [13], passive delivery of anti-M2e antibodies effectively protected mice from lethal challenge of influenza viruses, demonstrating the great potential of anti-M2e antibodies to treat influenza A virus infections [13,22,23]. To this end, anti-M2e mAbs were regarded as attractive candidates to treat influenza A virus infections.

In this study, the rational design of the epitope was based on the two considerations: (1) The first 26 nucleotides of influenza A genome segment seven are employed to code both M1 and M2 proteins, resulting the first nine amino acids in the N terminus of both proteins are identical and highly conserved. (2) Only six single mutations were identified in this region (Fig. 2). Herein, we generated mAb M2e8-7 targeting this epitope SLLTEVET (aa 2–9) and characterized its binding activity to M2e and the 21 M2e variant peptides and its inhibiting efficacy to different influenza A subtypes. As expected, the results showed that mAb M2e8-7 strongly cross reacted with M2e and 19 M2e variant peptides (Fig. 2), which was similar to another mAb Z3G1 described by Wang et al. [13]. MAb Z3G1 recognizes epitope LLTEVETPIR (aa 3–12), which overlaps seven residues of epitope of mAb M2e8-7 (Fig. 1). The influence of residue Serine in the N terminus of mAb M2e8-7 epitope and the residues Proline-Isoleucine-Arginine in the C terminus of mAb Z3G1 epitope to the function of the two mAbs need to be accessed.

Our data shed new light on the mechanism function of anti-M2e Abs in vitro. Previously, four mAbs were well defined for their therapeutic potential. The first reported mAb 14C2, which involved residue 11 and 14 of M2e, significantly reduced replication of A/Udorn/72 virus in the lungs of mice [23,24]. The other two mAbs 8C6 and 1B12, both recognized EVETPIRN epitope (aa 6–13), protected 75% mice from five LD50 (50% lethal dose) challenge of influenza virus A/PR/8/34 [22]. The latest described mAb Z3G1 which recognized epitope LLTEVETPIR (aa 3–12, Fig. 1), efficiently protected mice from lethal infection of A/HK/1/68 at different time regime [13]. Regardless of their protective efficacy in animal model, no in vitro study was described for mAbs 8C6, 1B12 and Z3G1. Our binding studies and in vitro assay clearly show that mAb M2e8-7 potently inhibited the replication of influenza A virus and anti-M2e mAbs displayed broad spectrum inhibiting effect against various influenza A subtypes in vitro.

More importantly, our results show that the two important amino acids in M2e epitope, Threonine at position five and the Glutamic acid at position six, were confirmed to lead antibody-escaping variants. Although only five strains and two subtypes published, the two escaped variants M2eV21 and M2eV22 which contain mutations at T5P and E6G, respectively represents two important genotypes emerged in Hong Kong and Africa in the last decade (Fig. 2). It will be essential to understand the mechanism of escaping and the resolution to prevent these variants. A comprehensive study on the cross reactivity of anti-M2e8T5P and anti-M2e8E6G mAbs, as well as changes in the mAbs Fv domains is ongoing in our laboratory. Another approach employed in our laboratory is to use phage display technique to compare the differences of peptide ligands to M2e8, M2e8T5P, and M2e8E6G epitopes.

The precise mechanism of mAb M2e8-7 remains to be determined. M2 is a minor transmembrane protein that functions as the virion proton channel. Inhibition of the virus replication by anti-M2e Abs is unlikely through blocking the entry or release of the virion. It is most possibly through blocking the ion channel directly in the extracellular part of M2 protein, a function similar to the adamantanes. MAb M2e8-7 may be very useful tool to study the mechanism function of M2 protein and can be used as a therapeutic agent against influenza A virus infections.

Acknowledgments

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