て高い増殖能が得られたワクチン用種ウイルスの増殖能の変化の原因について、アミノ酸変異の解析を行うことによって検討を行うと共に、ワクチン用種ウイルスから実際に不活化全粒子ワクチンを作製し、マウスへ経鼻接種を行った場合での抗ウイルス防御効果の誘導の是非について検討を行った。

B. 研究方法

1. インフルエンザウイルスの培養細胞への馴化および馴化株のアミノ酸置換の解析

北大・喜田 宏博士より御供与頂いたインフルエンザライブラリーの株16株をMDCK細胞中に17回継代を行い、10^8pfu/ml以上の高い増殖能を有する馴化ウイルス株を得た。そのうち、A/duck/Hokkaido/Vac-3/2007株（H5N1）のMDCK細胞馴化株について遺伝子解析を行い、馴化前後のアミノ酸置換の有無を検討した。

2. A/duck/Hokkaido/Vac-3/2007株のMDCK細胞馴化株を用いた不活化全粒子ワクチンの作製

A/duck/Hokkaido/Vac-3/2007株（H5N1）のMDCK細胞馴化株をMDCK細胞で培養させることにより10^{11} pfuまで増殖させ、スクロース比重遠心法によりウイルス粒子の精製を行った。精製されたウイルスに最終濃度1mg/ml になるように0.02%ホルマリン含有PBSを添加し、4℃下で28日間静置し、ウイルス粒子を固定させた。その後、透析にて固定液をPBSに置換させたものを不活化全粒子ワクチンとして用いた。なお、28日間のホルマリン固定によりウイルスのMDCK細胞への感染は認められないことを確認した。

3.マウスへの免疫およびウイルス攻撃試験

BALB/cマウス（6週齢、雌）にインフルエンザ不活化全粒子ワクチン（WVワクチン）を3週間間隔で2回経鼻接種した。WVワクチンの最終経鼻接種から2週間後に高病原性トリインフルエンザを発症した患者から分離された株であるA/HongKong/483/97株（H5N1）を致死量経鼻感染し、感染後のマウスの体重および生存率の変化を観察した。

C. 研究結果

1. トリインフルエンザウイルスA/duck/Hokkaido/Vac-3/2007株のMDCK細胞への馴化に伴うアミノ酸置換

インフルエンザライブラリー中の株であるA/duck/Hokkaido/Vac-3/2007 株は、MDCK細胞に17回継代することにより、増殖能が約10倍以上増強した（図1）。そこで我々は、MDCK細胞での馴化によるウイルスのアミノ酸置換について検討を行った。その結果、表１に示す通り、PB2の145番目、472番目、632番目、さらにPAの319番目のアミノ酸が置換されていることを明らかにした。

2. 試作用不活化全粒子ワクチンの経鼻接種による抗ウイルス防御効果

次にA/duck/Hokkaido/Vac-3/2007株を用いて試作用のホルマリン不活化WVワクチンを作製した。そして、WVワクチンをマウスに1μgを2回経鼻接種させ、最終接種から2週間後に致死量（100×LD_{50}）のA/HongKong/483/97株を感染させ、感染後のマウスの体重変化および生存率の変化を観察した。ワクチン非接種群において、感染後に体重減少がみられ、全匹死亡したが、ワクチン接種群においては、体重減少も見られず、全匹生存した（図2）。

D. 考察

昨年度の本研究において、ウイルスライブラリーに存在する株の大部分を継代することにより、MDCK細胞に馴化することを明らかにした。そして今回我々は、そのうちの1株であるA/duck/Hokkaido/Vac-3/2007株（H5N1）について、MDCK細胞での馴化による増殖能の増強に関与すると考えられるウイルス遺伝子の変異について検討した。その結果、PAおよびPB2の変異が増殖増強に関与することを明らかにした。他の血清型のウイルス株について、上記ウイルス株と同様もしくは類似の変異によって増殖能の増強がみられるか否かについて、現在検討を進めている。

ウイルスライブラリーからMDCK細胞に馴化させたA/duck/Hokkaido/Vac-3/2007株は、2つの遺伝子分節に変異がみられることから、この変異が免疫原性にどのような影響を示すか否かが重要な問題となる。今回我々は、MDCK細胞に馴化させたA/duck/Hokkaido/Vac-3/20株にホルマリン固定を行うことによりWVワクチンを作製し、同ワクチンの経鼻接種による抗ウイルス防御効果を確認し、2つの分節での遺伝子変異が免疫原性に影響を有しないことを明らかにした。さらに、このワクチン接種によって1997年に香港において高病原性トリインフルエンザを発症した患者から分離したA/HongKong/483/97株（H5N1）に対する感染防御効果を示した。このことは、MDCK細胞により馴化させた種ウイルスより作製したワクチンが実際に新たなインフルエンザパンデミックに即応し得ることを示すものであり、我々の進めている方法が新たなパンデミックに即応し得る迅速なインフルエンザワクチン作製の新たな手段になり得ることを強く示唆した。

E. 結論

1. H5N1型のトリインフルエンザウイルス株をMDCK細胞に馴化させることによるウイルス増殖能の増強にPB1およびPAの4つのアミノ酸置換が関与することを明らかにした。

2. MDCK細胞に馴化し高い増殖能を得た種ウイルスから作製した不活化全粒子ワクチンの経鼻接種は、高い抗ウイルス防御効果を誘導することを見出した。

F. 研究発表

1.論文発表

1. Okamoto S, Yoshii H, Matsuura M, Kojima A, Ishikawa T, Akagi T, Akashi M, Takahashi M, Yamanishi K, Mori Y. Poly-γ-glutamic acid nanoparticles and aluminum adjuvant used as an adjuvant with a single dose of Japanese encephalitis virus-like particles provide effective protection from Japanese encephalitis virus. Clin Vaccine Immunol 18:17-22, 2012.

2. Okamoto S, Matsuoka S, Takenaka N, Haredy AM, Tanimoto T, Gomi Y, Ishikawa T, Akagi T, Akashi M, Okuno Y, Mori Y, Yamanishi K: Intranasal immunization with formalin-inactivated human influenza A whole-virion vaccine alone and with split-virion vaccine with mucosal adjuvants shows similar cross-protection Clin Vaccine Immunol In press.

2.学会発表

1. 岡本成史、松岡須美子、Ahmad M. Haredy、山田博司、谷本武史、五味康行、石川豊数、奥野良信、赤木隆美、明石 満、森 康子、山西弘一. インフルエンザ不活化全粒子ワクチンの経鼻接種による交叉防御効果と抗ウイルス中和抗体産生との関連性. 第 25 回インフルエンザ研究者交流の会シンポジウム (2011 年 6 月 2-4 日 富山県富山市)

2. 岡本成史、谷本武史、高野大輔、松岡須美子、Ahmad M. Haredy、竹中延之、田村愼一、奥野良信、森 康子、山西弘一. 抗インフルエンザ IgA モノクローナル抗体による交叉防御効果の可能性. 第 15 回日本ワクチン学会学術集会 (2011 年 12 月 10-11 日 東京都千代田区)

3. 松岡須美子、岡本成史、Ahmad M. Haredy、谷本武史、五味康行、石川豊数、奥野良信、森 康子、山西弘一. インフルエンザ不活化全粒子ワクチンの経鼻接種による交叉防御効果の範囲と抗ウイルス中和抗体との関連性. 第 15 回日本ワクチン学会学術集会 (2011 年 12 月 10-11 日 東京都千代田区)

4. Okamoto S, Yamada H, Matsuoka S, Haredy AM, Tanimoto T, Gomi Y, Ishikawa T, Akashi M, Okuno Y, Mori Y, Yamanishi K. Intranasal immunization with formalin inactivated influenza A whole-virion vaccine alone induces Ssufficient cross-protection, correlating with cross-reactive neutralizing antibody production. International Union of Microbiological Societies 2011 Congress, Sep 11 - Sep 16, 2011 (Sapporo, Japan)

H. 知的財産権の出願・登録状況

なし。

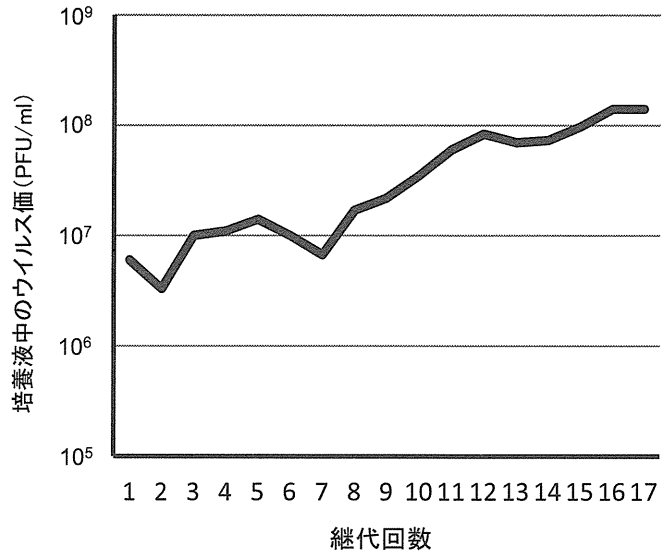

図1 MDCK 細胞への継代培養による A/duck/Hokkaido/Vac-3/2007 株(H5N1)の増殖能の変化。各代での培養上清中のウイルス価をプラーク法により測定した。

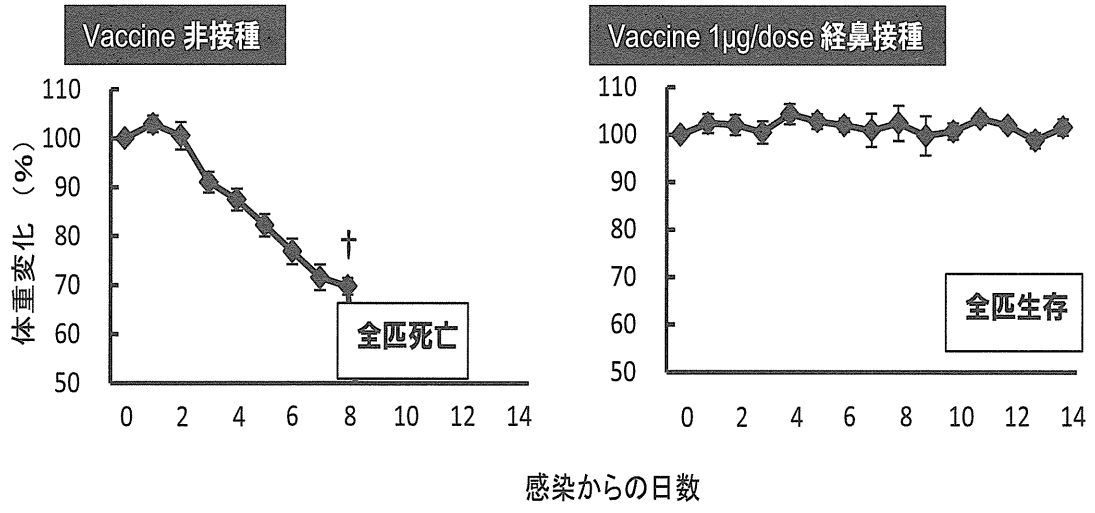

図2 A/duck/Hokkaido/Vac-3/2007 由来の WV ワクチンの経鼻接種による A/HongKong/483/97 の致死的感染に対する防御効果。WV ワクチンを2回経鼻接種し、その後致死量の A/HongKong/483/97 株を経鼻感染し、感染後の体重変化を観察した。

厚生労働科学研究費補助金（医療技術実用化総合研究事業）
分担研究報告書

サルを用いた経鼻ワクチンの安全性評価

研究分担者　内山安男　　順天堂大学大学院　　教授

研究要旨

本研究の目的ですが、感染予防を目的としたインフルエンザワクチンの一つに経鼻ワクチンがある。現在使われている皮下接種型のインフルエンザワクチンは、発症予防及び重篤化予防を目的としていて、感染そのものを予防することを目的としてはいない。今回、この経鼻ワクチンで侵入したウイルス粒子が神経細胞である、嗅上皮細胞に取り込まれるのか、また周辺の神経系に影響するのかを形態学的に解析し、挿入した不活化全粒子が、実際に、上皮に取り込まれ、どのような経路で処理されるのか形態学的に検討した。

A. 研究目的

インフルエンザウイルスは上気道炎を中心とする急性呼吸器症状及び小児における脳症を引き起す。同ウイルスはオルソミキソウイルス科（Orthomyxoviridae）に分類され、主にA型及びB型が知られている。現行のインフルエンザワクチンは、皮下接種型のワクチンであり、発症予防及び重篤化予防を目的としていて、感染そのものを予防することを目的としてはいない。近年、高病原性鳥インフルエンザウイルス（H５N１）がヒトに感染し致死的な病気を起こすことが報告されている。同ウイルス感染は、パンデミック（大流行）になる可能性も指摘されており、それに備えることは必須である。皮下ワクチン接種を引き起す危険性が危惧されており、それらに備えたワクチンの開発が急務となっている。さらに、ウイルスが鼻粘膜を介して侵入する場合、上皮を介して免疫系にどのように伝わるのかを検討することは重要である。

私達は、不活化全粒子ウイルスを鼻粘膜に投与し、その後の経過を形態学的に解析した。

本研究では、不活性か全粒子ウイルス（３種混合）をマカクの鼻腔に投与し、その後の経緯を免疫組織化学法と電子顕微鏡法を用いて解析し、侵入したウイルスが抗原提示細胞、リンパ球に伝達される経路を検討した。

B. 研究方法

１）用いた動物：本研究には、成獣マカクを７頭用いた。

２）インフルエンザ不活化全粒子ワクチン３種混合（A/カリフォルニア、A/ビクトリア、

B/ブリスベン）を 0.2 mL（50μg HA/dose）鼻腔に投与。神経系への影響を見るため、上鼻道に投与液が残留するように１０分間頭位を下げて放置した。

３）固定：マカクは、投与３、６，１２、２４時間後に光学顕微鏡用と電子顕微鏡用に固定した。固定は、光学顕微鏡レベルの免疫組織化学法用に４％パラフォルムアルデヒド（0.1 M phosphate buffer, pH 7.2）で、電子顕微鏡用には 2%グルタールアルデヒド-2%パラフォルムアルデヒド（0.1 M phosphate buffer, pH 7.2）溶液で灌流固定した。

４）光学顕微鏡試料：固定後、該当する鼻粘膜領域と嗅上皮領域を含む領域を採取し、さらに２４時間同固定液中に浸漬した。その後、１０%EDTA 溶液（pH 7.2、約１週間、冷暗所）で脱灰した。光学顕微鏡レベルの免疫組織化学法には、嗅上皮を含む領域、と一般鼻粘膜領域に別けて、１０%、２０%、３０%の蔗糖溶液に順次浸漬して凍結防止処理を施した後、Tissue-Tek OCT compound に包埋して、ドライアイス粉末で凍結した。凍結試料は１０μm 厚で、連続切片を作成した。連続切片の中から、一部をヘマトキシリン-エオシン（H-E）染色を施し、場所の同定を行なった。

５）電子顕微鏡資料の作成：灌流固定後、該当する鼻粘膜領域と嗅上皮領域を採取し、さらに２４時間同固定液に浸漬した。固定後、光学顕微鏡用試料と同様に１０%EDTA 溶液（pH 7.2、約１週間、冷暗所）で脱灰した。脱灰後、小片に細切し、脱水し、エポキシ樹脂に包埋した。包埋した資料をウルトラミクロトーム（UC6, Leica microsystems）で準超薄切片（１μm 厚）を作成して、上皮の種類を検討した。

６）免疫染色：３種類の不活化全粒子に対する抗体を検討した。本研究では、特に、抗 AH3 型ウイルス HA 蛋白抗体(U1-37)を一次抗体として用いて染色した。二次抗体にはマウス IgG に対する蛍光あるいは HRP 標識抗体を用いた。蛍光標識した切片は、必用に応じて各種血球（顆粒球、マクロファージ）に対する抗体、嗅上皮特異抗体を用いて二重標識し、核染色（DAPI）後、蛍光退色防止剤入り封入剤に封入した。観察は、オリンパスあるいは Zeiss 共焦点顕微鏡で行なった。また、HRP 標識に対しては、DAB 処理した後、ヘマトキシリンで軽く核染色した後、封入して観察に供した。

C. 研究結果と考察

現在、光学顕微鏡用の全試料を連続凍結切片として作製を終えた。これら切片を用いて、抗体の反応性を検討している。

電顕試料についても、全試料の準超薄切片の作成を実施して、嗅粘膜上皮と一般粘膜上皮が各時点で採取できていることの確認を終えている。

凍結切片や電子顕微鏡用試料の作成は十分に行なえ、今後の解析に耐え得る試料であることは確認できた。今後、これらの試料を用いてウイルス粒子の侵入経路を検討する予定である。

２．実用新案登録

該当無し

３．その他

該当無し

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Okamoto,S., Matsuura,M., Akagi,T., Akashi,M., Tanimoto,T., Ishikawa,T., Takahashi,M., Yamanishi,K., Mori,Y.,	Poly(gamma-glutamic acid)nano-particles combined with mucosal influenza virus hemagglutinin vaccine Protects against influenza Virus infection in mice	Vaccine	27(42)	5896-5905	2009
Itoh, Y., Ozaki, H.,Ishigaki, H., Sakoda, Y., Nagata,T., Soda, K., IsodaN., Miyake, T., Ishida, H., Okamoto, K., Nakayama,M., Tsuchiya, H., Torii, R., Kida, H., and Ogasawara, K	Subcutaneous inoculat-ion of a whole virusparticle vaccine preparedfrom a non-pathogenic virus library induces protective immunity against H7N7 highly pathogenic avian influenza virus in cynomolgus macaques.	Vaccine	28	780-789	2010

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Itoh, Y., Shinya, K., Kiso, M., Watanabe, T., Sakoda, Y., Hatta, M., Muramoto, Y., Tamura, D., Sakai-Tagawa, Y., Noda, T., Sakabe, S., Imai, M., Hatta, Y., Watanabe, S., Li, C., Yamada, S., Fujii, K., Murakami, S., Imai, H., Kakugawa, S., Ito, M., Takano, R., Iwatsuki-Horimoto, K., Shimojima, M., Horimoto, T., Goto, H., Takahashi, K., Makino, A., Ishigaki, H., Nakayama, M., Okamatsu, M., Warshauer, D., Shult, P. A., Saito, R., Suzuki, H., Furuta, Y., Yamashita, M., Mitamura, K., Nakano, K., Nakamura, M., Brockman-Schneider, R., Mitamura, H., Yamazaki, M., Sugaya, N., Suresh, M., Ozawa, M., Neumann, G., Gern, J., Kida, H., Ogasawara, K., and Kawaoka, Y.	In vitro and in vivo characterization of new sine-origin H1N1 influenza viruses.	Nature	460	1021-1025	2009
Kashima, Y., Ikeda, M., Itoh, Y., Sakoda, Y., Nagata, T., Miyake, T., Soda, K., Ozaki, H., Nakayama, M., Shibuya, H., Okamatsu, M., Ishigaki, H., Ishida, H., Sawai, T., Kawaoka, Y., Kida, H., and Ogasawara, K.	Intranasal administration of alive non-pathogenic avian H5N1 influenza virus from a virus library confers protective immunity against H5N1 highly pathogenic avian influenza virus infection in mice: Comparison of formulations and administration routes of vaccines.	Vaccine	27	7402-7408	2009
Manzoor, R., Sakoda, Y., Nomura, N., Tsuda, Y., Ozaki, H., Okamatsu, M., and Kida, H.	PB2 protein of a highly pathogenic avian influenza virus strain A/chicken/Yamaguchi/7/2004 (H5N1) determines its replication potential in pigs.	J Virol	83	1572-1578	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Miyake, T., Soda, K., Itoh, Y., Sakoda, Y., Ishigaki, H., Nagata, T., Ishida, H., Nakayama, M., Ozaki, H., Tsuchiya, H., Torii, R., Kida, H., and Ogasawara, K.	Amelioration of pneumonia with Streptococcus pneumoniae infection by inoculation with a vaccine against highly pathogenic avian influenza virus in a non-human primate mixed infection model.	J Med Primatol	39	58-70	2009
Moritoh, K., Yamauchi, H., Asano, A., Yoshii, K., Kariwa, H., Takashima, I., Isoda, N., Sakoda, Y., Kida, H., Sasaki, N., and Agui, T.	Generation of congenic mouse strains by introducing the virus-resistant genes, Mx1 and Oas1b, of feral mouse-derived inbred strain MSM/Msi nto the common strain C57BL/6J.	Jpn J Vet Res	57	89-99	2009
Sasaki, T., Isoda, N., Soda, K., Sakamoto, R., Saijo, K., Hagiwara, J., Kokumai, N., Ohgitani, T., Imamura, T., Sawata, A., Lin, Z., Sakoda, Y., and Kida, H.	Evaluation of the potency, optimal antigen level and lasting immunity of inactivated avian influenza vaccine prepared from H5N1 virus.	Jpn J Vet Res	56	189-198	2009
Sasaki, T., Kokumai, N., Ohgitani, T., Sakamoto, R., Takikawa, N., Lin, Z., Okamatsu, M., Sakoda, Y., and Kida, H.	Long lasting immunity in chickens induced by a single shot of influenza vaccine prepared from inactivated non-pathogenic H5N1 virus particles against challenge with a highly pathogenic avian influenza virus.	Vaccine	27	5174-5177	2009
Simulundu, E., Mweene, A. S., Tomabechi, D., Hang'ombe, B. M., Ishii, A., Suzuki, Y., Nakamura, I., Sawa, H., Sugimoto, C., Ito, K., Kida, H., Saiwana, L., and Takada, A.	Characterization of H3N6 avian influenza virus isolated from a wild white pelican in Zambia.	Arch Virol	154	1517-1522	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsuda, Y., Isoda, N., Sakoda, Y., and Kida, H.	Factors responsible for plaque formation of A/duck/Siberia/272/1998 (H13N6) influenza virus on MDCK cells.	Virus Res	140	194-198	2009
Yoshida, R., Igarashi, M., Ozaki, H., Kishida, N., Tomabechi, D., Kida, H., Ito, K., and Takada, A.	Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses.	PLoS Pathog	5	e1000350	2009
Hasegawa H, Ichinohe T, Ainai A, Tamura S, Kurata T.	Development of an inactivated mucosal vaccine for H5N1 influenza virus.	Ther Clin Risk Manag	Feb; 5(1)	125-32	2009
Takahashi Y, Hasegawa H, Hara Y, Ato M, Ninomiya A, Takagi H, Odagiri T, Sata T, Tashiro M, Kobayashi K.	Protective immunity afforded by H5N1 (NIBRG-14)-inactivated vaccine requires both antibodies against hemagglutinin and neuraminidase in mice.	J Infect Dis	Jun 1; 199(11)	1629-37	2009
Ichinohe T, Ainai A, Tashiro M, Sata T, Hasegawa H.	PolyI:polyC12U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants.	Vaccine	Oct 23; 27(45)	6276-9	2009
Ichinohe T, Ainai A , Nakamura T , Akiyama Y, Maeyama J, Odagiri T , Tashiro M, Takahashi H, Sawa H, Tamura S, Chiba J, Kurata T, Sata T, Hasegawa H.	Induction of cross-protective immunity against influenza A virus H5N1 by intranasal vaccine with extracts of mushroom mycelia.	J Med Virol	82	128-137	2010
Ainai A, Ichinohe T, Tamura S, Kurata T, Sata T, Tashiro M, Hasegawa H.	Zymosan enhances the mucosal adjuvant activity of Poly(I:C) in a nasal influenza vaccine.	J Med Virol	Mar; 82(3)	476-84	2010
Tamura S, Hasegawa H, Kurata T.	Estimation of the effective doses of nasal -inactivated influenza vaccine in humans from mouse-model experiments.	Jpn J Infect Dis	Jan; 63(1)	8-15	2010

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakajima N, Hata S, Sato Y, Tobiume M, Katano H, Kaneko K, Nagata N, Kataoka M, Ainai A, Hasegawa H, Tashiro M, Kuroda M, Odai T, Urasawa N, Ogino T, Hanaoka H, Watanabe M, Sata T.	The First Autopsy Case of Pandemic Influenza (A/H1N1pdm) Virus Infection in Japan: Detection of a High Copy Number Of the Virus in Type II Alveolar Epithelial Cells by Pathological and Virological Examination.	Jpn J Infect Dis	Jan; 63(1)	67-71	2010
Takiyama A, Wang L, Tanino M, Kimura T, Kawagishi N, Kunieda Y, Katano H, Nakajima N, Hasegawa H, Takagi T, Nishihara H, Sata T, Tanaka S.	Sudden Death of a Patient with Pandemic Influenza (A/H1N1pdm) Virus Infection by Acute Respiratory Distress Syndrome.	Jpn J Infect Dis	Jan; 63(1)	72-4	2010
Ohno, S., S. Kohyama, M.Taneichi, O. Moriya, H. Hayashi, H. Oda, M. Mori, A. Kobayashi, T. Akatsuka, T. Uchida, and M. Matsui.	Synthetic peptides coupled to the surface of liposomes effectively induce SARS coronavirus-specific cytotoxic T lymphocytes and viral clearance in HLA-A*0201 transgenic mice.	Vaccine	27	3912-3920	2009
Kohyama, S., S. Ohno, T. Suda, M. Taneichi, S. Yokoyama, M. Mori, A. Kobayshi, H. Hayashi, T. Uchida, and M. Matsui.	Efficient induction of cytotoxic T lymphocytes specific for severe acute respiratory syndrome (SARS)-associated coronavirus by immunization with surface-linked liposomal peptides derived from a non-structural polyprotein 1a.	Antiviral Res	84	168-177	2009
Matsui, M., S. Kohyama, T. Suda, S. Yokoyama, M. Mori, A. Kobayashi, M. Taneichi, and T. Uchida.	A CTL-based liposomal vaccine capable of inducing protection against heterosubtypic influenza viruses in HLA-A*0201 transgenic mice.	Biochem. Biophys. Res. Commun.	391	1494-1499	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Takagi, A., M. Matsui, S.Ohno, H. Duan, O. Moriya, N. Kobayashi, H. Oda, M. Mori, A. Kobayashi, M. Taneichi, T. Uchida, and T. Akatsuka	Highly efficient anti-viral $CD8^+$ T cell induction by peptides coupled to the surface of liposomes.	Clin. Vaccine Immunol	16	1383-1392	2009
内田哲也、種市麻衣子	インフルエンザと抗原	メディカル・サイエンス・ダイジェスト	35	566-567	2009
内田哲也、種市麻衣子	リポソームを用いた感染症ワクチンの開発	DDS	25	29-36	2010
内田哲也、種市麻衣子	季節性及び新型インフルエンザに有効な CTL誘導型リポソームワクチン	ファルマシア	46	119-123	2009
内田哲也、種市麻衣子	新発想のインフルエンザワクチン：細胞性免疫誘導型インフルエンザワクチンの開発	化学	64	26-29	2010
Mori,H., Yamanaka,K., Matsuo,K., Yasutomi,Y. And Mizutani,H	Administration of Ag85B showed therapeutic effects to Th2-type cytokine-mediated acute phase atopic dermatitis by inducing regulatory T cells.	Arch. Dermatol.Res.	301	151-157	2009
Okabayashi, S., Ohno,C. and Yasutomi,Y.	Acute megakaryocytic leukemia (AMKL)-like disease in a Cynomolgus monkey (Macaca fascicularis).	J.Comp.Pathol.	140	212-216	2009
Morioka ,T., Yamanaka,K., Mori,H., Omoto,Y., Tokime, K., Kakeda,M., Kurokawa,I., Gabazza,E., Tsubura A., Yasutomi,Y. and Mizutani, H	IL-4/IL-13 antagonist DNA vaccination successfully suppresses Th2 type chronic dermatitis.	Br.J.Dermatol.	160	1172-1179	2009
Takano, J.I., Tachibana, H., Kato, M., Narita, T., Yanagi, T., Yasutomi, Y. and Fujimoto	DNA characterization of simian Entamoeba histolytica-like strains to differentiate them from Entamoeba histolytica.	Parasitol.Res.	105	929-937	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yasuhiro Yasutomi	Establishment of specific pathogen-free macaque colonies in Tsukuba Primate Research Center of Japan for AIDS research	Vaccine	In press		2009
Fujimoto,K., Takano,J., Narita,T., Hanari,K., Shimozawa,N., Sankai,T., Yoshida T., Terao,K., Kurata ,T. and Yasutomi,Y.	Simian Retrovirus type D infection in a colonyof cynomolgus monkeys.	Comp. Med.	In press		2009
Cueno,M.E., Karamatsu,K., Yasutomi, Y., Laurena,A.C. and Okamoto.T.	Preferential expression and immunogenicity of HIV-1 Tat fusion protein expressed in tomato plant.	Transgenic. Res.	In press		2010
Uchdida,A., Sasaguri,H., Kimura,N., Tajiri,M., Ohkubo, T., Ono,F., Sakaue,F.,Kanai, K., Hirai,T., Sano,T., Shibuya,K., Kobayashi,M., Yamamoto,M., Yokota,S.., Kubodera,T., Tomori,M., Sakaki, K., Enomoto,M., Hirai,Y., Kumagai,J., Yasutomi,Y., Mochizuki,H., Kuwabara,S., Uchihara,T., Mizusawa,H. and Yokakota,T.	Non-human primate model of ALS with cytoplasmic mislocalization of TDP-43.	Brain			印刷中
Iwasaki,Y., Mori,K., Ishii,K., Maki,N., Iijima,S., Yoshida,T., Okabayashi,S., Katakai ,Y., Lee,J., Saito,A., Fukai, H., Kimura,N., Ageyama,N., Yoshizaki,S., Suzuki,T., Yasutomi,Y., Miyamura,T., Kannagi,M. and Akari,H.	Longe-term persistent GBV-B infection and development of a chronic and progressive hepatitis C-like diseae in marmosets. Frontiers Microbiol.	Frontiers Microbiol.			印刷中

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hirata,H., Kawai,S., Maeda, M., Jinnai,M., Fujisawa,K., Katakai,Y., Hikosaka,K.,Tanabe,K., Yasutomi,Y. and Ishihara,C.	Identification and Phylogenetic Analysis of Japanese Macaque Babesia-1 (JM-1) Detected from a Japanese Macaque (Macacafuscata fuscata).	Am.J.Trop.Med.Hyg.			印刷中
Saito,A., Kono,K., Nomaguchi,M., Yasutomi,Y., Adachi,A., Shioda,T., Akari,H. and Nakayama,E.E.	Geographic, Genetic, and Functional Diversity of AntiretroviralHost Factor TRIMCyp in Cynomolgus Macaque (Macaca fascicularis)	J.Gen.Virol.			印刷中
Matsuo,K. and Yasutomi,Y.	Mycobacterium bovis bacille Calmette-Guérin as a vaccine vector for global infectious disease control.	Tuberculosis Res. Treat.	Epub		2011
Chono,H., Saito,N.. Yasutomi,Y.,Mineno,J., and Kato,I.	In vivo safety and persistence of endoribonuclease gene-transduced CD4+ T cells in cynomolgus macaques for HIV-I gene therapy model.	PloS One	Epub		2011
Xing, Li., Wang, J.C., Li, T-C., Yasutomi,Y., Lara,J., Purcell,R., Takeda,N., Miyamura,T. and Holland,R.C.	Spatial configuration of hepatitis E virus antigenic domain.	J.Virol	85	1117-1124	2011
Chono,H., Matsumoto,K., Tsuda,H., Saito,N., Lee,K.., Kim,S., Shibata,H., Ageyama,N., Terao,K., Yasutomi,Y., Mineno J., Kim,S., Inoue, M. and Kato,I.	Acquistion of HIV-1 resistance in T lymphocytes using an ACA-specific E.coli mRNA interferase.	Human Gene Ther.	22	35-43	2011
Okabayashi,S., Uchida,K., Ohno,C., Hanari,K., Goto,I. and Yasutomi,Y.	Periventricular Leucomalacia(PVL)-like lesions in two neonatal cynomolgus monkeys (macaca fascicularis).	J.Comp.Pathol	144	204-211	2011

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Saito,A., Nomaguchi,M., Iijima,S.,Lee,Y-J., Kono,K., Nakayama,E.E., Shioda,T., Yasutomi,Y., Adachi,A., Matano,T., Akari,H.	A novel monkey-tropic HIV-1 derivative encoding only minimal SIV sequences can replicate in cynomolgus monkeys.	Micorbes and Infection	13	58-64	2011

【参考資料】

Journal of Virology

Enhanced Growth of Influenza Vaccine Seed Viruses in Vero Cells Mediated by Broadening the Optimal pH Range for Virus Membrane Fusion

Shin Murakami, Taisuke Horimoto, Mutsumi Ito, Ryo Takano, Hiroaki Katsura, Masayuki Shimojima and Yoshihiro Kawaoka

J. Virol. 2012, 86(3):1405. DOI: 10.1128/JVI.06009-11.
Published Ahead of Print 16 November 2011.

JVI
Journals.ASM.org

Enhanced Growth of Influenza Vaccine Seed Viruses in Vero Cells Mediated by Broadening the Optimal pH Range for Virus Membrane Fusion

Shin Murakami,[a]* Taisuke Horimoto,[a,b] Mutsumi Ito,[a] Ryo Takano,[a,c] Hiroaki Katsura,[a] Masayuki Shimojima,[a] and Yoshihiro Kawaoka[a,c,d,e]

Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan[a]; Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan[b]; ERATO Infection-Induced Host Responses Project, Japan Science and Technology Agency, Saitama, Japan[c]; International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan[d]; and Influenza Research Institute, Department of Pathological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin, USA[e]

Vaccination is one of the most effective preventive measures to combat influenza. Prospectively, cell culture-based influenza vaccines play an important role for robust vaccine production in both normal settings and urgent situations, such as during the 2009 pandemic. African green monkey Vero cells are recommended by the World Health Organization as a safe substrate for influenza vaccine production for human use. However, the growth of influenza vaccine seed viruses is occasionally suboptimal in Vero cells, which places limitations on their usefulness for enhanced vaccine production. Here, we present a strategy for the development of vaccine seed viruses with enhanced growth in Vero cells by changing an amino acid residue in the stem region of the HA2 subunit of the hemagglutinin (HA) molecule. This mutation optimized the pH for HA-mediated membrane fusion in Vero cells and enhanced virus growth 100 to 1,000 times in the cell line, providing a promising strategy for cell culture-based influenza vaccines.

Although several antivirals against influenza viruses, including neuraminidase (NA) inhibitors, have been developed and used worldwide, vaccination is still considered one of the most effective preventive measures to combat influenza (12, 23). Currently, most conventional influenza vaccines are produced from viruses grown in embryonated chicken eggs. However, the limited capacity of the egg-dependent vaccine supply could be problematic in terms of securing enough doses when facing a pandemic situation, such as occurred in 2009, or in the event of a pandemic originating from a highly pathogenic avian virus, such as an H5N1 virus. In these situations, cell culture-based systems could play an important role for robust vaccine production (4).

Presently, cell culture-based inactivated influenza vaccines are in clinical trials or have been approved for use in some countries (1, 7, 8, 13, 19). This approach has considerable advantages over egg-based vaccines because (i) it can lead to more rapid and larger-scale vaccine production (10); (ii) it may avoid the potential for selecting variants adapted for chicken eggs, which alters virus antigenicity (18); (iii) selection of high-yield vaccine seed viruses is needed for egg-based production; and (iv) it does not contain allergic components of eggs (16). Due to these advantages, the World Health Organization (WHO) has recommended the establishment of mammalian cell culture-based vaccines (41).

Several cell lines are currently approved for cell culture-based influenza vaccine production. One of them, the African green monkey Vero cell line, has a good track record for the production of other viral vaccines for human use (e.g., polio and rabies vaccines) (26). In their long history, Vero cells have proven safe for vaccine production, so the WHO now recommends this cell line as an alternative substrate for influenza vaccine production (2). However, since seed viruses for seasonal inactivated vaccines occasionally grow suboptimally in Vero cells, seed viruses that grow well in Vero cells must be carefully selected for robust vaccine production (37). Here, we present a strategy for the development of vaccine seed viruses with enhanced growth in Vero cells by changing an amino acid residue in the hemagglutinin (HA) stem region. This approach could help overcome shortages in the influenza vaccine supply in emergency pandemic situations.

MATERIALS AND METHODS

Cells. African green monkey Vero WCB cells, approved for use in human vaccine production (38), were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) with 10% fetal calf serum and antibiotics. Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (MEM) with 5% newborn calf serum and antibiotics. The cells were maintained at 37°C in 5% CO_2.

Virus adaptation to Vero cells. The A/Puerto Rico/8/34 [PR8(UW)] strain (27, 31) was generated by using reverse genetics (29) and propagated in 10-day-old embryonated chicken eggs for 2 days at 37°C, after which the allantoic fluids containing viruses were harvested and stored at −80°C. PR8 virus was inoculated into Vero cells in bovine serum albumin (BSA) (0.3%)-containing MEM with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (1 μg/ml). Three to 4 days after infection, virus-containing supernatants were collected and inoculated into fresh Vero cells at 1:100 or 1:1,000 dilution. After 11 passages, virus-containing supernatant was collected and stored at −80°C. Stock virus titers were determined by using a plaque assay in MDCK cells.

Received 15 August 2011 Accepted 8 November 2011
Published ahead of print 16 November 2011
Address correspondence to Taisuke Horimoto, ahorimo@mail.ecc.u-tokyo.ac.jp, or Yoshihiro Kawaoka, kawaoka@ims.u-yokyo.ac.jp.
* Present address: Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, USA.

doi:10.1128/JVI.06009-11

TABLE 1 Comparison of amino acid sequences between wild-type PR8 and PR8-Vero viruses

Virus gene	Accession no.	Amino acid position	Residue	
			PR8 WT	PR8-Vero
HA (HA2)	AB671289[a]	117	N	D (4)[b]
NA	AB671290	255	N	Y (4)
PB2	AB671295	740	D	N (2)

[a] GenBank accession number of wild-type sequence.
[b] Number of clones that acquired the mutation out of four clones.

Virus gene sequencing. Viral RNAs were extracted from supernatants by using a commercial kit (QiaAmp viral RNA isolation kit; Qiagen) and were converted to cDNAs by using reverse transcriptase (SuperScript III; Invitrogen) and primers based on the consensus sequences of the 3-prime ends of the RNA segments for the H1N1 viruses. The full-length cDNAs were then PCR amplified with *Pfu*Ultra DNA polymerase (Stratagene) and PR8-specific primer pairs for each segment. The amplified cDNAs were cloned by using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). For each segment, four clones were sequenced by using PR8-specific primers. Primer sequences are available upon request.

Construction of mutant viruses. We used our previously produced series of PolI constructs, derived from PR8(UW), for reverse genetics (15, 27) and PolI plasmids containing the HA and NA genes derived from A/Kawasaki/173/2001 (H1N1; Kawasaki173; GenBank accession numbers AB671296 and AB671297 for HA and NA, respectively), A/Kawasaki/UTK-4/09 (H1N1; UTK-4; GenBank accession numbers AB671291 and AB671292 for HA and NA, respectively), A/California/04/09 (H1N1; CA04; GenBank accession numbers FJ966082.1 and FJ966084.1 for HA and NA, respectively), and A/Yokohama/2013/03 (H3N2; Yok2013; GenBank accession numbers AB671293 and AB671294 for HA and NA, respectively) (20, 30). To generate HA, NA, and PB2 mutants, PolI plasmids expressing the HA, NA, and PB2 genes of PR8, Kawasaki173, UTK-4, CA04, or Yok2013 were used as templates for site-directed mutagenesis by the inverse PCR method. PR8 mutants and PR8 backbone 6:2 reassortants containing the six internal segments of PR8 and the HA and NA segments of seasonal or pandemic viruses were generated by using reverse genetics (29).

Virus replication in Vero and MDCK cells. Virus was inoculated into Vero or MDCK cell monolayers at a multiplicity of infection (MOI) of 0.01 PFU/cell with MEM containing BSA and 1.0 μg/ml TPCK-trypsin and incubated at 37°C (for PR8, PR8/Kawasaki173 6:2 reassortant, PR8/UTK-4 6:2 reassortant, PR8/CA04 6:2 reassortant, and PR8/Yok2013 6:2 reassortant viruses) or at 33°C (for PR8/CA04 6:2 reassortant viruses). Viruses in the culture supernatants were collected at a given number of hours postinfection (p.i.) and then titrated by use of an MDCK plaque assay to determine the virus titers.

Cell fusion assay. The cell fusion assay was performed as previously described (33) with some modifications. Briefly, Vero cells were transfected with PR8 HA or mutant HA (N117DHA2) expression plasmids (pCAGGS-PR8HA or pCAGGS-PR8HA2N117D, respectively), as well as with a green fluorescent protein (GFP) expression plasmid (pCAGGS-GFP) to conveniently visualize fused cells under a fluorescence microscope. After transfection, the cells were incubated at 37°C for 24 h. The cells were then washed several times with Mg^{2+}- and Ca^{2+}-containing phosphate-buffered saline (PBS^-) and treated with 5 μg/ml of TPCK-trypsin for 5 min at 37°C. The trypsin was then inactivated by washing with PBS^+ containing FCS. To initiate cell fusion, the cells were treated with acidic PBS (adjusted with citric acid) for 1 min and then incubated in FCS-containing medium at 37°C for 30 min. Fused cells were observed under a fluorescence microscope (Biozero; Keyence).

Comparison of endosomal pHs. Comparisons of endosomal pHs between Vero and MDCK cells were performed as previously described (25, 35), with some modifications. Briefly, Vero and MDCK cells were incubated with Alexa Fluor 647 (30 μg/ml; Invitrogen)- and Oregon green 488 (250 μg/ml; Invitrogen)-conjugated dextran and incubated for 15 min at 37°C. After incubation, the cells were immediately placed on ice and washed 5 times with ice-cold PBS^+, and the intensities of the Alexa Fluor 647 and Oregon green 488 were measured by using confocal microscopy (LSM 510; Carl Zeiss) in five microscopic fields for each sample. The Oregon green 488/Alexa Fluor 647 intensity ratio was then calculated.

Statistical analysis. All comparisons of the infectivity titers of each virus and the intensity ratio for Oregon green 488/Alexa Fluor 647 relied on Student's *t* test with two-tailed analysis to determine significant differences.

RESULTS

Adaptation of PR8 virus for Vero cells. To obtain a virus that grows to a high titer in Vero cells, we performed serial passages of the PR8 virus in the cell line. Initially, wild-type (WT) PR8 virus-infected Vero cells showed an ambiguous cytopathic effect. After eight passages, however, we observed a clear cytopathic effect in Vero cells. After the 11th passage, we collected the virus (referred to as the PR8-Vero virus). We then compared wild-type and PR8-Vero virus titers in the supernatants of infected Vero cells. Wild-type virus grew to 2.0×10^4 PFU/ml, whereas PR8-Vero virus grew to 1.9×10^9 PFU/ml. These data suggest that PR8-Vero virus possesses mutations that enhance its replication in Vero cells.

Identification of mutations responsible for Vero cell adaptation. To identify the mutation(s) responsible for PR8 adaptation to Vero cells, the virus genome was sequenced. The cDNAs of the PR8-Vero virus were cloned into plasmids, and the sequences of four clones for each segment were read. As shown in Table 1, PR8-Vero virus contained mutations in the HA (4/4 clones), NA (4/4 clones), and PB2 (2/4 clones) genes, and all of these mutations caused amino acid changes. No mutations were identified in any of the other gene segments. Next, we introduced the mutation(s) into wild-type PR8 by using reverse genetics and examined the growth kinetics of the mutants (Fig. 1). The D740N PB2 PR8 mutant virus grew similarly to wild-type PR8, whereas the N255Y NA mutation augmented viral growth to 8.5×10^5 PFU/ml. Interestingly, the N117D HA2 mutant virus grew to the highest titer (6.3×10^8 PFU/ml). Both the N255Y NA and N117D HA2 mutations caused virus to grow to a level comparable to that of PR8-

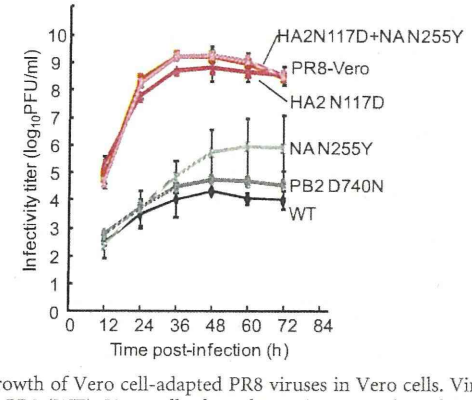

FIG 1 Growth of Vero cell-adapted PR8 viruses in Vero cells. Viral titers of wild-type PR8 (WT), Vero cell-adapted PR8 (PR8-Vero), and mutants possessing amino acid substitution(s) observed in PR8-Vero viruses were determined at 12, 24, 36, 48, 60, and 72 h p.i. at an MOI of 0.01. The data are reported as mean titers with standard deviations for three independent experiments.

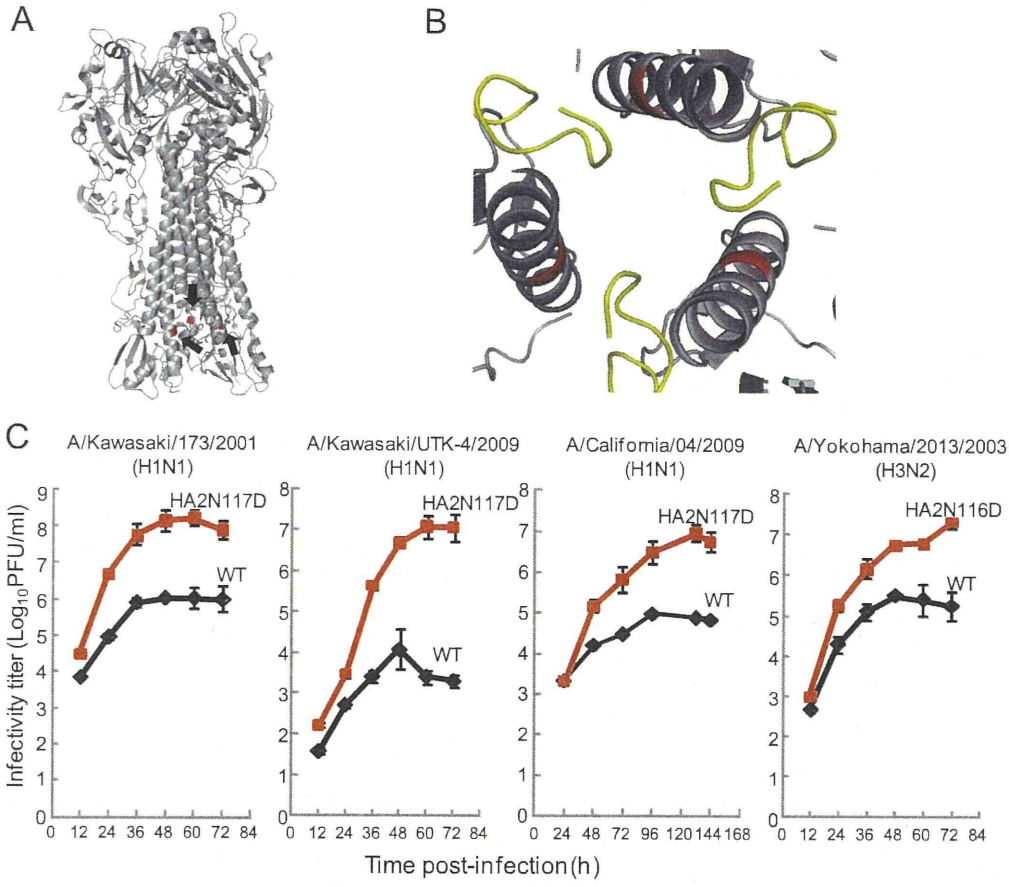

FIG 2 Growth enhancement of vaccine seed-like viruses mediated by the HA2 N117D (N116D) mutation. (A) Location of the HA2 N117 residue on the three-dimensional (3D) structure of A/Puerto Rico/8/34 (PDB:1RU7). HA2 117 is colored red. (B) Close-up view of the region indicated by arrows in panel A. The view is from the globular head of the HA trimer. The locations of HA2 N117 and fusion peptides are colored as follows: HA2 117, red; fusion peptide, yellow. (C) Growth of PR8 background 6:2 reassortant viruses possessing the HA2 N117D (N116D for H3) mutation. The viral titers of the parental 6:2 reassortant (WT) and mutants were determined at the indicated times after infection at an MOI of 0.01. The data are reported as mean titers with standard deviations for three independent experiments.

Vero virus. These data demonstrate that the N117D HA2 mutation is primarily responsible for Vero cell adaptation.

Generation of a high-growth vaccine seed virus in Vero cells. Based on a National Center for Biotechnology Information (NCBI) database search, more than 99% of viruses possess asparagine at position 117 (H1 subtype) or 116 (H3 subtype) of HA2. Asparagine at this position is located in the α-helix of the stalk region of the HA molecule, which is close to the viral membrane surface (Fig. 2A). We therefore sought to determine whether the HA2 N117D (or N116D) amino acid substitution could enhance the growth of other viruses in Vero cells. To this end, we introduced the HA2 N117D or N116D mutation into the HAs of the H1 seasonal strains A/Kawasaki/173/01 (H1N1) and A/Kawasaki/UTK-4/09 (H1N1), the pandemic strain A/California/4/2009 (H1N1), and the H3 seasonal strain A/Yokohama/2013/2003 (H3N2). We then generated PR8/H1N1 or PR8/H3N2 6:2 reassortants with these mutated HAs by using reverse genetics. We also produced 6:2 reassortants with wild-type HA from each virus for comparison. Each virus possessing mutated HA grew to a 100 to 1,000 times higher titer than its wild-type HA-bearing counterpart in Vero cells (Fig. 2C). In contrast, we did not observe any marked differences in virus titers between wild-type and mutant reassortants in MDCK cells (data not shown). These data suggest that the HA2 N117(116)D mutation would universally enhance the growth of a vaccine seed virus in Vero cells.

The HA2 N117D mutation alters the pH range for virus membrane fusion. Since the HA2 region mediates virus membrane fusion and the HA2 N117 residue is located close to the fusion peptide (Fig. 2B), we examined whether the mutation affects the optimal pH for viral membrane fusion. We constructed plasmids expressing PR8 wild-type HA or HA2 N117D mutant HA and transfected them into Vero cells. A GFP expression plasmid was cotransfected into the cells for a fusion assay. At 24 h posttransfection, we treated the cells with several low-pH buffers and observed fused cells (Fig. 3). At neutral pH (pH 7.4), we did not observe any fused cells with either wild-type HA or N117D mutant HA-transfected Vero cells. At pH 5.0 and 5.2, numerous fused cells were observed in both wild-type HA and mutant HA-transfected cells. Interestingly, at pH 5.4, few fused cells were observed in wild-type HA-transfected cells, whereas many fused cells were observed in mutant N117D HA-transfected cells. At pH 5.6, both wild-type HA and mutant HA-transfected cells produced limited cell-cell fusion. In addition to the change in optimal pH for membrane fusion in mutant HA-expressed fused cells, the