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Measurement of Antibodies to Varicella-Zoster Virus Using a Virus-Free Fluorescent-Antibody-to-Membrane-Antigen (FAMA) Test

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The fluorescent-antibody-to-membrane-antigen (FAMA) test is regarded as the "gold standard" to detect protective antibodies to varicella-zoster virus (VZV) because of its high sensitivity and specificity. Because the classic FAMA test uses an infectious virus for detection of antibodies to VZV, it is labor-intensive, and also requires special equipment for handling the virus. For this reason, we attempted to develop a simple and safe FAMA assay. Because VZV glycoprotein E (gE) is one of the major VZV glycoproteins, we used the gE protein for the FAMA test (gE FAMA). Here, we demonstrate that overexpression of gE in HEK293T cells can be used to measure antibodies in human serum, and that gE FAMA titers are closely correlated with gpEIA ELISA data. These results indicate that our gE FAMA test has the potential to measure antibodies to VZV.

Keywords: Varicella-zoster virus, fluorescent-antibody-to-membrane-antigen test, chickenpox, shingles, glycoprotein E

Introduction

Varicella-zoster virus (VZV), a member of the human alpha herpesvirus family, causes chickenpox (varicella) and shingles (zoster); VZV is highly specific in that it infects only humans [18]. The presence of antibodies to VZV in human serum reflects a history of VZV infection, and also protects against VZV infection [1, 13]. Therefore, serological tests for the presence of VZV antibodies are useful to determine the susceptibility of individuals to VZV. Serological tests with low sensitivity may result in superfluous vaccinations, while tests with low specificity may produce false-positive results [1]. For this reason, a sensitive and specific method should be used to determine immune status.

Currently, several methods to measure VZV antibody are available; however, none of these methods are both simple and accurate. The fluorescent-antibody-to-membrane-antigen (FAMA) test is the assay used most extensively to measure the presence of protective VZV antibodies because of its high sensitivity and specificity [9, 17]. A FAMA titer ≥1:4 correlates with protection from VZV infection with some exceptions [1, 4, 13]. Although the FAMA test is regarded as the "gold standard," it is labor-intensive, and also requires special equipment for both virus infection and protection to virus exposure. An alternative method to measure antibodies to VZV is an enzyme-linked immunosorbent assay (ELISA). Reported VZV ELISAs used VZV-infected cell lysates or purified glycoproteins [1, 14, 16]. However, these commercial ELISAs are less sensitive than the classic FAMA VZV assay [1]. Furthermore, the expression pattern of viral proteins, for example punctuated staining in the cytoplasm, can be obtained from the FAMA test, and can provide additional information to exclude false-positives

caused by nonspecific interactions.

The major viral antigens of VZV are structural glycoproteins, namely glycoprotein E (gE), glycoprotein B (gB), glycoprotein H (gH), and glycoprotein L (gL) [11, 12]. VZV gE is the most abundant viral glycoprotein in VZV-infected cells, and VZV gB is the second most abundant glycoprotein [5]. VZV gH is the third most abundant glycoprotein, and is associated with VZV gL [3]. Because VZV gE is the most abundant glycoprotein, VZV gE has been used to detect antibodies using a luciferase immunoprecipitation assay system [2, 15]. Here, we developed a modified FAMA test using overexpressed gE protein, and named this test gE FAMA. Because our gE FAMA test does not require infectious virus particles, it is safe and procedurally simple to perform. We also compared gE FAMA titers with gpEIA ELISA data for verification.

Materials and Methods

Cell Culture, Infection, and Transfection

HEK293A, HEK293T, and MRC-5 cells were grown in DMEM (Welgene, Korea) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). MRC-5 cells were infected with VZV as described previously [6, 7]. Transfection of HEK293T cells was carried out using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA).

cDNA Cloning

PCR was used to amplify the entire coding region of VZV gE from genomic DNA isolated from VZV-infected MRC-5 cells using the forward primer 5′-ATG GGG ACA GTT AAT AAA CC-3′ and the reverse primer 5′-TCA CCG GGT CTT ATC TAT ATA C-3′. Genomic DNA was prepared using the Hirt extraction method [8]. Amplified cDNA fragments were cloned into pcDNA4/HisMax (Invitrogen) and full-length sequences were obtained.

Human Serum

For gE FAMA analysis, a total of 22 human plasma samples were used. This work was approved by the Institutional Review Board of Yeungnam University Medical Center (IRB No. YUH-13-0330-O16). Obtained plasma was stored at −70°C until use.

Western Blotting

Cells were harvested and lysed with lysis buffer (0.15 M NaCl, 50 mM HEPES (pH 8.0), 0.5% NP40) containing a protease inhibitor cocktail (Roche, Germany). For western blot analysis, polypeptides in whole-cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane filters. Detection was conducted with a 1:2,000 or 1:5,000 dilution of WHO international standard for VZV immunoglobulin (NIBSC W1044, UK) using an enhanced chemiluminescence (ECL) system. Images were acquired using a Chemidoc-it 410 imaging system (UVP, Upland, CA, USA) and LAS4000 system (GE Healthcare, Uppsala, Sweden). HRP-conjugated anti-human antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

FAMA Test

For the FAMA test, cells were infected either with VZV or plasmid. Basically, we performed the classic FAMA test according to William's method with some modifications [10, 17]. For gE FAMA, HEK293T cells were transfected with plasmid encoding VZV gE. Twenty-four hours after transfection, cells were washed and incubated in trypsin-EDTA solution for 2 min, and then cells were resuspended in fresh DMEM. Cells were then incubated with diluted human plasma for 30 min and subsequently incubated with FITC-conjugated anti-human antibody for 30 min. Finally, cells were washed and mounted on a glass slide in mounting medium (Vector, Burlingame, CA, USA). Images were captured with a Carl Zeiss LSM710 confocal microscope (Oberkochem, Germany). FITC-conjugated anti-human antibody was purchased from Santa Cruz Biotechnology.

gpEIA ELISA Test

gpEIA ELISA was performed to measure the antibody titer in human plasma. Antibody titer was measured using the Serion ELISA Classic VZV IgG kit (Serion/Viron, Germany) according to the manufacturer's instructions [14]. Briefly, this kit measures IgG antibodies specific to viral envelope glycoproteins and the detection range is 15 to 2,000 mIU/ml. Samples beyond the detection limit (>2,000 mIU/ml) were diluted 10-fold and then re-analyzed.

Results

gE is Immunogenic and Effective in the FAMA Assay

Herpes viral glycoproteins are responsible for immunogenicity, and we attempted to identify the major glycoprotein responsible for the humoral response. We cloned the VZV glycoproteins gE, gH, and gB, and examined the response to the international standard VZV human immunoglobulin. We used VZV-infected MRC-5 cell lysates as a positive control. Human anti-VZV immunoglobulin produced multiple bands, with a strong band at around 70 kDa. Next, we examined protein expression by western blotting, and we found that glycoprotein E (gE) produced a strong signal and had a size close to that of the strong band observed in VZV-infected cell lysates (Figs. 1A and 1B). We also examined the expression of gH and gB, but their expressions were low and not suitable for the FAMA assay (data not shown). These results indicate that VZV gE is highly immunogenic.

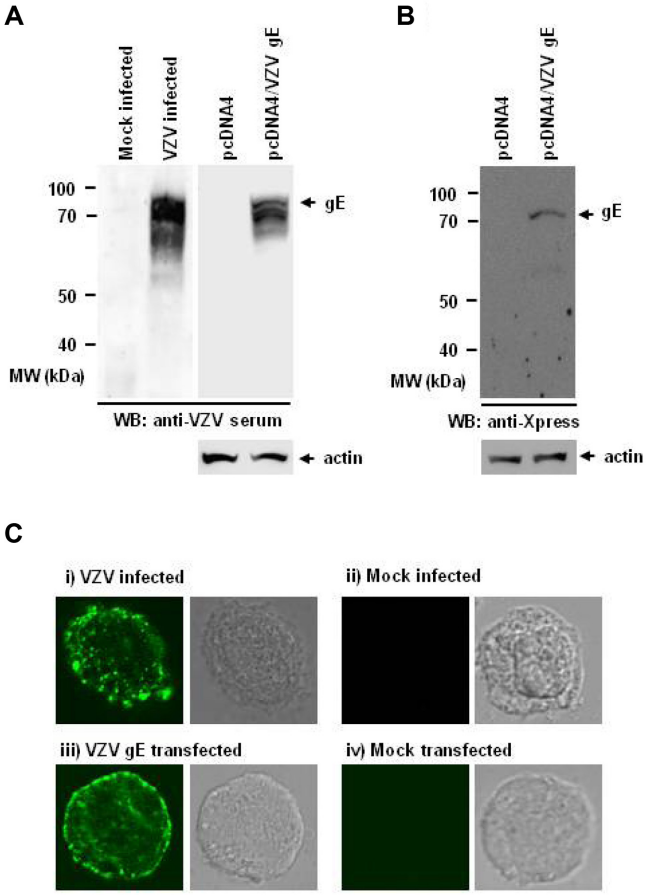

Fig. 1. Expression of VZV glycoprotein E (gE).
(**A**) VZV gE is one of the major VZV antigens. MRC-5 cells were infected with VZV, and HEK293T cells were transfected with the plasmid encoding VZV gE. Equal amounts of cell lysates were subjected to western blotting with international standard VZV immunoglobulin. (**B**) Confirmation of VZV gE expression. HEK293T cells were transfected with the plasmid encoding VZV gE. Equal amounts of cell lysates were subjected to western blotting with anti-Xpress antibody. (**C**) FAMA staining of VZV-infected cells and gE transfected cells. HEK293T cells were either infected with VZV (i) or transfected with gE (iii). Twenty-four hours after treatment, cells were stained with VZV immunoglobulin (100 mIU/ml). Mock infected cells (ii) and mock transfected cells (iv) were stained as a negative control.

Because of the strong immunogenicity of VZV gE, we performed a FAMA assay with overexpressed gE protein (gE FAMA assay). HEK293T cells were transfected with plasmid encoding VZV gE. Twenty-four hours after transfection, cells were detached and incubated with 100 mIU/ml of international standard VZV immunoglobulin. Whereas mock transfected cells did not stain, a strong signal was obtained for gE-transfected cells (Fig. 1C). VZV-infected cells were used as a positive control; the gE FAMA signal was comparable to FAMA performed with VZV-infected cells (classic FAMA) (Fig. 1C). In particular, the staining pattern of the gE FAMA test was close to that of the classic FAMA test (Fig. 1C).

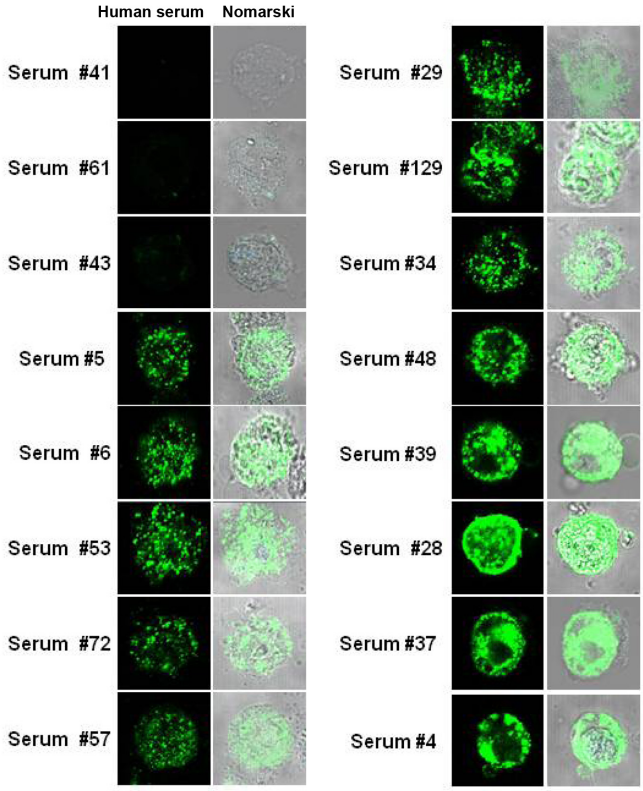

Fig. 2. Confocal microscope image of HEK293T cells transfected with VZV gE.
HEK293T cells were transfected with a plasmid encoding VZV gE. Twenty-four hours after transfection, cells were stained with 50-fold diluted human plasma and microscope images were obtained using a LSM710 confocal microscope.

gE FAMA is Specific and Sensitive

Because we obtained similar results for gE FAMA and classic FAMA, we examined the presence of antibodies to VZV in 22 human serum samples. HEK293T cells transiently transfected with gE plasmid were incubated with 50-fold diluted sera, and the stained cells were analyzed by confocal microscopy. A punctuated or ring-shaped staining pattern was observed, which can be used for specific and sensitive detection (Fig. 2). The signal intensity differed among plasma samples (Fig. 2). Antibody titer was also measured with a glycoprotein ELISA (gpEIA) kit. The signal

A

Sample #	mIU/ml	FAMA titration										
		4	8	16	32	64	128	256	512	1024	2048	4096
41	2.99	-										
61	40.9	+	-									
43	41.87	+	-									
5	129.48	+	+	+	+	-						
6	220.45	+	+	+	+	+	-					
33	275.11	+	+	+	+	+	-					
53	365.8	+	+	+	+	+	-					
72	496.53	+	+	+	+	+	-					
57	529.7	+	+	+	+	+	+	-				
29	619.72	+	+	+	+	+	+	-				
129	622.2	+	+	+	+	+	+	+	-			
34	747.39	+	+	+	+	+	+	+	-			
70	963.36	+	+	+	+	+	+	-				
56	1132.74	+	+	+	+	+	+	+	+	-		
26	1159.39	+	+	+	+	+	+	+	+	-		
80	1329.51	+	+	+	+	+	+	+	+	-		
48	1799.57	+	+	+	+	+	+	+	+	-		
13	1873.35	+	+	+	+	+	+	+	+	+	-	
39	2134.15	+	+	+	+	+	+	+	+	+	-	
28	2163.86	+	+	+	+	+	+	+	+	+	+	-
37	2376.83	+	+	+	+	+	+	+	+	+	+	-
4	2478.46	+	+	+	+	+	+	+	+	+	+	-

B

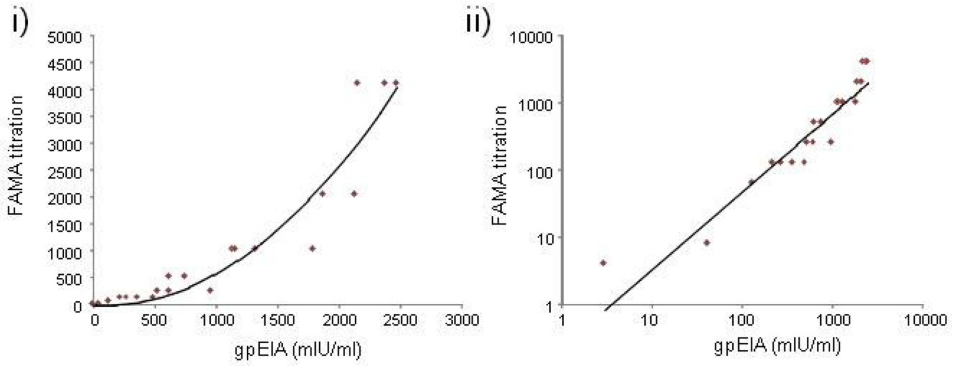

Fig. 3. Correlation between gpEIA and gE FAMA titers of 22 human serum samples.
(**A**) gE FAMA titration results. HEK293T cells were transfected with the plasmid encoding gE, and cells were immunostained with serially diluted human sera. The "+" indicates presence of signal, and "-" indicates absence of signal. (**B**) Correlation between gpEIA results and gE FAMA. Correlations are shown on a linear scale (i) and log scale (ii). (Correlation coefficient (R^2) = 0.9024).

intensity of gE FAMA correlated with the gpEIA antibody titer (Fig. 2). These results indicate that gE FAMA can be used to diagnose the presence or absence of VZV antibodies.

Correlation Between gpEIA and gE FAMA Titers

Because gE FAMA specifically detects VZV antibodies in human plasma, we measured the amount of VZV antibody using titration. Human plasma samples were serially diluted and used for FAMA staining. Cells were immunostained with serially diluted sera, and those with a specific signal were marked "+", while those that had no signal were marked "-". The results of all plasma panels tested are summarized in Fig. 3A. Because gpEIA can quantitate the amount of antibody to VZV, we compared the gE FAMA titer with the gpEIA antibody titer. As expected, the gE FAMA titer was strongly positively correlated with the gpEIA titer (R^2 = 0.9024) (Fig. 3B). These results indicate that gE FAMA can be used to quantify VZV antibody levels.

Discussion

In this report, we described a modified FAMA test to measure antibodies to VZV. Owing to its high sensitivity and specificity, the FAMA test is the "gold standard" used to measure the presence of protective VZV antibodies, especially vaccine immunity. However, the classic FAMA test uses infectious virus, and special equipment is required to prevent the virus from spreading to the surroundings. Furthermore, investigators are at risk of virus infection

during preparation of VZV-infected cells. To establish a simple and safe FAMA test, we overexpressed VZV gE in HEK293T cells, which are easy to transfect and express high levels of plasmid-encoded proteins. We tested other cell lines, including HEK293A, but we did not get sufficient gE expression for the FAMA test (data not shown). Because our modified gE FAMA test does not require infectious virus or an infection procedure, it is more cost-effective than the classic FAMA test. Although gE FAMA requires transient transfection of cells, many easy transfection methods are available, and our gE FAMA method can be easily adapted in other labs.

Staining patterns of the gE FAMA test were similar to those of the classic FAMA test. When the antibody titer was low, punctuated stained spots were evident in the cytoplasm of cells. However, a high antibody titer resulted in ring-shaped staining in the cytoplasm as well as cytoplasmic dots. These staining patterns can be used to exclude false-positive results while retaining the advantages of the classic FAMA test. Another advantage of our test is that while expression of viral proteins and virus particle production have a cytopathic effect on virus-infected cells, gE-expressing cells remain morphologically normal regardless of length of time after transfection.

Because VZV serological tests are performed to determine susceptibility to VZV infection, the cutoff value is important. A titer of <1:4 serum dilution and a gpEIA <50 mIU/ml have been used as negative cutoff values [1, 10]. gE FAMA titration revealed that FAMA negative at 1:8 was lower than gpEIA 50 mIU/ml. Because gE FAMA is based on a single overexpressed protein, the expression level of gE is different from that in virus-infected cells, and the cutoff value likely differs between the classic FAMA and gE FAMA. Further experiments to determine the cutoff value of gE FAMA for susceptibility to VZV infection are required.

Although the VZV gpEIA can be automated for high-throughput screening, the FAMA test is the reference test for all other methods. The FAMA test is the most sensitive test developed thus far, and the staining pattern can provide additional information about specificity and preciseness. During our experiment, we often observed aggregates of proteins or antibodies with fluorescence. This kind of false-positive signal cannot be detected in ELISAs. A FAMA assay should be performed to confirm the presence of antibodies to VZV; the gE FAMA test described in this paper is another good option owing to its simplicity and safety. Further evaluation of the characteristics of the FAMA test, including whether it can be automated, is required for wide application of gE FAMA. High content analysis or cellomics devices can potentially be used for the automated analysis of FAMA results.

Acknowledgments

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