Selection of Vaccinia Virus-Neutralizing Antibody from a Phage-Display Human-Antibody Library

Yong Won Shin^{1*}, Ki-Hwan Chang¹, Gwang-Won Hong¹, Sang-Gu Yeo², Youngmee Jee², Jong-Hyun Kim^{2,3}, Myoung-don Oh⁴, Dong-Hyung Cho⁵, and Se-Ho Kim^{6,7*}

¹R&D Center, GC Pharma, Yongin 16924, Republic of Korea

²Division of VPD Control and NIP, Korea Centers for Disease Control and Prevention, Ministry of Health and Welfare, Osong Health Technology Administration Complex, Cheongju 28159, Republic of Korea

³Department of Pediatrics, The Catholic University of Korea College of Medicine, St. Vincent's Hospital, Suwon 16247, Republic of Korea

⁴Department of Internal Medicine, College of Medicine, Seoul National University, Seoul 03080, Republic of Korea

⁵School of Life Science, Kyungpook National University, Daegu 41566, Republic of Korea

⁶Division of Biomedical Convergence, College of Biomedical Science, Kangwon National University, Chuncheon 24341, Republic of Korea

⁷Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon 24341, Republic of Korea

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*Corresponding authors

S.-H.K.

Phone: +82-33-250-7364;

Fax: +82-33-250-8380;

E-mail: kimsho@kangwon.ac.kr

Y.W.S.

Phone: +82-31-260-9024;

Fax: +82-31-260-1864;

E-mail: ywshin@greencross.com

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Although smallpox was eradicated in 1980, it is still considered a potential agent of biowarfare and bioterrorism. Smallpox has the potential for high mortality rates along with a major public health impact, eventually causing public panic and social disruption. Passive administration of neutralizing monoclonal antibodies (mAbs) is an effective intervention for various adverse reactions caused by vaccination and the unpredictable nature of emerging and bioterrorist-related infections. Currently, vaccinia immune globulin (VIG) is manufactured from vaccinia vaccine-boostered plasma; however, this production method is not ideal because of its limited availability, low specific activity, and risk of contamination with blood-borne infectious agents. To overcome the limitations of VIG production from human plasma, we isolated two human single-chain variable fragments (scFvs), (SC34 and SC212), bound to vaccinia virus (VACV), from a scFv phage library constructed from the B cells of VACV vaccine-boostered volunteers. The scFvs were converted to human IgG1 (VC34 and VC212). These two anti-VACV mAbs were produced in Chinese Hamster Ovary (CHO) DG44 cells. The binding affinities of VC34 and VC212 were estimated by competition ELISA to IC₅₀ values of 2 µg/ml (13.33 nM) and 22 µg/ml (146.67 nM), respectively. Only the VC212 mAb was proven to neutralize the VACV, as evidenced by the plaque reduction neutralization test (PRNT) result with a PRNT₅₀ of ~0.16 mg/ml (~1.07 µM). This VC212 could serve as a valuable starting material for further development of VACV-neutralizing human immunoglobulin for a prophylactic measure against post-vaccination complications and for post-exposure treatment against smallpox.

Keywords: Monoclonal antibody, phage display, single-chain variable fragment (scFv), smallpox, vaccinia virus, vaccinia immune globulin

Introduction

Smallpox is caused by the variola virus (VARV), which belongs to the Orthopoxvirus genus. Vaccinia and cowpox viruses are also included in the same genus as VARV [1]. It

is known that cross-protection in the Orthopoxvirus genus is possible, and therefore smallpox can be prevented by vaccinia virus (VACV) vaccination [2].

The World Health Organization (WHO) declared that smallpox was eradicated in 1980 and smallpox vaccination

was discontinued in the early 1980s. However there exists the possibility of bioterror threat with VARV [3], and smallpox vaccination was reintroduced in response to this risk. Concomitantly, vaccinia immune globulin (VIG) was required for management and possible prophylaxis for vaccine-related serious adverse events [4].

Currently VIG is prepared from plasma with high titers of anti-vaccinia immunoglobulin. The donors for plasma are boosted with a VACV vaccine to raise the titers of anti-vaccinia immunoglobulin [5]. However, the production of VIG from vaccinated human plasma entails inconvenience in collection. In addition, the availability of high-titer plasma is limited, and there exists the possibility of contamination with infectious agents [6].

VACV-specific human monoclonal antibodies (mAbs) can replace VIG, which can eliminate the risks typically associated with human blood products and provide a well-characterized, high-quality immune globulin [6]. Several experimental approaches are currently available for generating human mAbs, and phage display is the most widely used technology for in vitro selection of antibodies [7]. With this approach, it is possible to select mAbs of virtually any specificity in vitro, thereby providing mAbs not only for therapeutic use in humans but also for use in research and clinical diagnostics [8].

In this study, a phage display library of human single-chain variable fragment (scFv) antibodies was constructed from the B cells of healthy volunteers boosted by the VACV vaccine. Human scFvs against VACV were then isolated, and their neutralizing efficacy was demonstrated following conversion to human immunoglobulin G1 (IgG1).

Materials and Methods

Blood collection and RNA isolation

Five healthy volunteers were vaccinated with a smallpox vaccine (Lancy-Vaxina vaccine; Berna Biotech, Switzerland) once. After 7 days of vaccination, 20 ml of blood was collected from each individual using a vacutainer tube (Becton Dickinson, USA) containing sodium heparin. Vaccination and blood collection were approved by the Institutional Review Board of Seoul National University Hospital (IRB No. H-0606-015-175).

Isolation of lymphocytes from the blood, preparation of total RNA, and cDNA synthesis were performed as previously described [9].

Construction of an antibody library and screening of anti-VACV scFvs

DNA was amplified using Ready-To-Go RT-PCR Beads (GE Healthcare Bio-Sciences, USA) according to the manufacturer's

Fig. 1. Arrangement of genes in the pKS4H vector.

LacZ denotes the lacZ promoter, and g3 denotes the leader peptide of gene III of filamentous phage M13. VH and VL denote variable regions of the immunoglobulin H and L chains, respectively. H denotes the 6× His tag. Gene III denotes the gene that encodes a minor coat protein of filamentous phage M13. Restriction enzyme sites used for cloning scFv are shown.

instructions. Primers were designed to fit into the N- and C-terminal regions of each variable heavy chain (VH), variable kappa chain (Vκ), and variable lambda chain (Vλ) region (Table 1). VHs were amplified with a mixture of primers JH1, JH2 and JH3 and with primers VH1, VH3, and VH4, respectively, followed by another round of polymerase chain reaction (PCR) with primers VH Ext and LINK2. Vκs were amplified with a mixture of primers JK1, JK2, and JK3 and with Vκ1/3 and Vκ2, followed by another round of PCR with primers Jκ Ext and LINK1. Vλs were amplified with a mixture of primers Jλ1 and Jλ2 and a mixture of primers Vλ1 and Vλ3, followed by another round of PCR with primers Jλ Ext and LINK1. Purification of PCR products, cloning into pKS4H (Fig. 1), and preparation of a phage-displayed antibody library were performed as previously described [9].

ScFvs that bind to VACV were screened by panning in immunotubes (Maxisorp; NUNC A/S, Denmark) as previously described [10]. Tubes were prepared by coating with 1 ml of Vaccinia (Lister strain) purified virus (Advanced Biotechnologies, Inc., USA) diluted up to 2.5 µg/ml in phosphate-buffered saline (PBS).

Expression of scFv in *Escherichia coli*, and ELISA to measure scFv binding to VACV

Expression of scFv in colonies obtained from the fourth round of panning and ELISA for measuring the binding of scFv to VACV were performed as previously described [11]. VACV was diluted to 2.5 µg/ml in PBS and immobilized in 96-well plates (Nunc Immuno Module, Maxisorp; NUNC A/S). ScFvs bound to VACV were detected using the 6x-His Epitope Tag Antibody, HRP conjugate (Thermo Fisher Scientific, USA) and 3,3',5,5'-tetramethylbenzidine (TMB) solution (KPL, USA) as a substrate. The sequencing of colonies displaying binding to VACV in ELISA was performed by Genotech (Korea) using primer P035 (Table 1). Two scFv clones, SC34 and SC212, were screened based on their binding to VACV and by sequence analysis for the next step.

Establishment of a cell line producing human anti-VACV IgG₁

For construction of the VH-expression vector, the signal

Table 1. Oligonucleotide primers used for cloning and sequencing of human immunoglobulin genes.

VH1:	CAG CCG GCC ATG GCC CAG GTG CAG CTG GTG CAG TCT GGG
VH3:	CAG CCG GCC ATG GCC SAG GTG CAG CTG GTG GAG TCT GGG
VH4:	CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GGC
JH1:	ACC TCC GCC TGA ACC GCC TCC ACC TGA GGA GAC RGT GAC CAG GGT
JH2:	ACC TCC GCC TGA ACC GCC TCC ACC TGA AGA GAC GGT GAC CAT TGT
JH3:	ACC TCC GCC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CGT GGT
VH Ext:	GTT GTT CCT TTC TAT <u>GCG GCC CAG CCG GCC</u> ATG GCC (<i>SfiI</i>)
Vκ1/3:	GGT GGC TCC GGA GGT GGC GGA TCG GAC ATC CAG ATG ACC CAG TCT CCA
Vκ2:	GGT GGC TCC GGA GGT GGC GGA TCG GAT ATT GTG ATG ACC CAG ACT CCA CTC
Jκ1:	TCG ACT TGC GGC CGC ACG TTT GAT WTC CAC YTT GGT CCC
Jκ2:	TCG ACT TGC GGC CGC ACG TTT GAT CTC CAS CTT GGT CCC
Jκ3:	TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC
Jκ Ext:	GAG TCA TTC TCG ACT <u>TGC GGC CGC</u> ACG TTT (<i>NotI</i>)
Vλ1:	GGT GGC TCC GGA GGT GGC GGA TCG CAG TCT GYS CTG ACT CAG CCA CCC
Vλ3:	GGT GGC TCC GGA GGT GGC GGA TCG TCC TAT GAG CTG ACW CAG CCA CCC
Jλ1:	TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT SAS CTT GGT CCC
Jλ2:	TTC TCG ACT TGC GGC CGC ACC GAG GAC GGT CAG CTG GGT GCC
Jλ Ext:	GAG TCA TTC TCG ACT <u>TGC GGC CGC</u> ACC (<i>NotI</i>)
LINK1:	GGT GGA GGC GGT TCA GGC GGA GGT GGC <u>TCC GGA</u> GGT GGC GGA TCG (<i>BspEI</i>)
LINK2:	CGA TCC GCC ACC <u>TCC GGA</u> GCC ACC TCC GCC TGA ACC GCC TCC ACC (<i>BspEI</i>)
P013:	GGG AGA CCC <u>AAG CTA GCT</u> CAG ACA GGC AG (<i>NheI</i>)
P023:	CAT CAC ACT <u>GGC GGC CGC</u> TCT AGA ACT (<i>NotI</i>)
P030a:	CCA GCT GCA CCT GAC TCT GGA CAC CAT T
P030:	AAT GGT GTC CAG AGT CAG GTG CAG CTG G
P031:	GAA GAC CGA <u>TGG GCC CTT</u> GGT GGA GGC TGA GGA GAC GGT G (<i>ApaI</i>)
P032a:	GAG TCA GCT CAT AGG ACC CAC AGG TAC CAG A
P032:	TCT GGT ACC TGT GGG TCC TAT GAG CTG ACT C
P033a:	GGG GTT GGC CTT GGG CTG ACC TAG GAC GGT GAG C
P033:	GCT CAC CGT CCT AGG TCA GCC CAA GGC CAA CCC C
P034:	GAA TAC <u>GGG CCC</u> CTA TTA TGA ACA TTC TGT AGG (<i>ApaI</i>)
P035:	CAA CGT GAA AAA ATT ATT ATT CGC

^aThe primer sequences are shown in the 5'-3' direction. S denotes G or C. W denotes A or T. Y denotes C or T. The restriction enzymes used for cloning are shown in parentheses, and their recognition sites are underlined.

sequence (Sig) of pRC13 (Fig. 2) [12] was amplified by PCR using primers P023 and P030a (all primers are listed in Table 1), and the VH sequence was amplified by PCR using primers P030 and P031 on the scFvs described above (SC212 and SC34). The two

amplified fragments (Sig and VH) were then fused by PCR using primers P023 and P031, and this Sig-VH PCR fragment was inserted into pRC13 following digestion with *NotI* and *ApaI*.

For construction of the variable light chain (VL) expression vector, Sig of pKC12 [12] was amplified by PCR using primers P013 and P032a (Fig. 2) [12], and the VL was amplified by PCR using primers P032 and P033a on SC212 and SC34. The two fragments (Sig and VL) were then fused by PCR using primers P013 and P033a. The lambda constant region (CL) was amplified by PCR using primers P033 and P034 from the cDNA of human B lymphocytes prepared for library construction. The CL PCR fragment was fused to the Sig-VL PCR fragment by PCR using primers P013 and P034, and this Sig-VL-CL PCR fragment was inserted into pKC12 following digestion with *NheI* and *ApaI*.

The CHO DG44 cell line expressing human anti-VACV IgG₁ was established as previously described [12]. The established cell lines VC34 and VC212 were adapted to serum-free medium P1 (GC Pharma, Korea), and antibodies were produced in a

Fig. 2. Arrangement of genes in expression vectors. pRC13 for heavy chain (A) and pKC12 for light chain (B). Restriction enzyme sites used for cloning are shown. *P_{CMV}*, cytomegalovirus promoter; *Sig*, signal peptide of human immunoglobulin.

disposable-wave bioreactor (GE Healthcare). The mAbs of VC34 and VC212 were purified from the culture media using protein A agarose beads (GE Healthcare), followed by ion-exchange chromatography and verification of antibody purity by SDS-PAGE.

Determination of affinities of selected clones

The affinities of anti-VACV mAbs (VC34 and VC212) were determined by competition ELISA, as described previously [13], using VACV-coated plates and free VACV. The binding affinity (IC_{50}) was defined as the VACV concentration that provides 50% inhibition of anti-VACV mAb binding to immobilized VACV as compared with maximum binding, *i.e.*, ELISA readings performed in the absence of competitive VACV.

Determination of VACV neutralization by anti-VACV mAb

VACV neutralization by anti-VACV mAb was determined by a plaque-reduction neutralization test (PRNT) [14]. For PRNT, 1×10^6 Vero E6 cells (Korea Centers for Disease Control and Prevention (KCDC), Korea) were seeded into 6-well plates and incubated overnight. The VACV_{WR} strain (KCDC) was diluted to 2 pfu/ μ l in Eagle's minimum essential medium (EMEM; Lonza, USA) supplemented with 1% fetal bovine serum (FBS). Anti-VACV mAbs were 2-fold serially diluted (up to 1:1024) with EMEM containing 1% FBS. The starting concentrations of mAb VC34 and mAb VC212 were 2.9 mg/ml and 2.4 mg/ml, respectively. Equal volumes (120 μ l) of diluted mAbs and VACV were then mixed and incubated at 37°C for 18 h.

The 6-well plates were washed twice with PBS, and 200 μ l of the virus/mAb mixture was added and incubated at 37°C in 5% CO₂ for 90 min. Medium was aspirated, and cells were overlaid with 2 ml of overlay medium [1:1 mixture of 2 $\times$ MEM containing 2% FBS and 1% agarose (SeaPlaque agarose; Lonza)] and incubated for 2 days. After the medium was aspirated, cells were fixed with 10% formaldehyde for 1 h, stained with crystal violet, and washed once with PBS, and plaques were then counted. The PRNT₅₀ was defined as the mAb concentration that caused a 50% reduction in the number of VACV plaques as compared with the number of VACV plaques obtained from culture media.

Results

Amplification of DNA and library construction

Total RNA was prepared from the peripheral blood of smallpox-vaccine-immunized healthy volunteers. A group of primers targeting the N- and C-termini of the VH and VL (V κ and V λ) chains was designed and synthesized, and the subsequent PCR products appeared at the expected size of ~350 bp by agarose gel electrophoresis (Fig. 3). The fragments were inserted into the phagemid vector pKS4H (Fig. 1), in which the lacZ promoter drives expression, and the g3 leader sequence guides secretion of scFvs. Gene III,

Fig. 3. Analysis of amplified PCR products.

Agarose gel (1.2%) analysis of PCR fragments of VH, V κ , and V λ chains. M: DNA ladder (Life Technologies, Carlsbad, CA, USA); lane 1: VH1; lane 2: VH3; lane 3: VH4; lane 4: V κ 1/3; lane 5: V κ 2; lane 6: V λ 1 and V λ 3.

which encodes the minor coat protein of filamentous phage M13, is positioned at the C-terminus of scFv, thereby allowing scFv to be displayed on the surface of the phage particles.

The library was constructed by inserting VH first, followed by VL, into the phagemid pKS4H, resulting in a library size of $\sim 1.0 \times 10^9$. Library diversity was confirmed by DNA sequencing of individual colonies from the library (data not shown).

Selection of scFvs that bind to VACV

For selection of anti-VACV scFvs, four rounds of panning were performed in the VACV-immobilized immunotubes, and scFv expression was induced in the colonies from the fourth round of panning. ScFvs were expressed in *E. coli* XL1 Blue cells and secreted into the culture medium, and ELISA was performed on VACV-immobilized plates to confirm the binding of the secreted scFvs to VACV. Two clones, SC34 and SC212, were selected, and their sequences analyzed (Fig. 4), revealing that both scFvs possessed V λ .

Conversion of scFv to human IgG1 and estimation of binding affinity

The VH and VL of SC34 and SC212 obtained from library screening were converted to human IgG₁ (mAb VC34 and mAb VC212) via insertion into the mammalian expression vectors pRC13 (VH) and pKC12 (VL), respectively. Because the original form of pKC12 contained the constant region of the kappa chain, the lambda constant region was cloned from the cDNA of B lymphocytes.

	FR1	CDR1	FR2	CDR2
34VH	QVQLVQSGGKLVKPGGSLRISCAASGFPLS	DHFMT	WVRQAPGKGLEWVS	YISYPSGNIKYYGDSMRG
212VH	-----GV-Q-R-----TFD	NFA-H	----V-----A	A---*D-TTEEST--VK-
	FR3	CDR3		
34VH	RFTVSRDNTKKSVYLQMSNLRAEDTAVYYCAR	VGGSVYADILTDWFD	WGPGTTVTVSS	
212VH	-----SQNRLF---NS--T-----K	GLTPLWFGVV*-A--V	--R--M-----	

	FR1	CDR1	FR2	CDR2	FR3
34VL	SYELTQPPSVSVAPGQTARITC	GGNNIGAKSVH	WYQQRPGQAPLLVVY	DDSDRPS	GIPERISGSNF
212VL	-----K--T-A--	--S---T---Q	----K-----I-	-HG--A-	-----F---KS
	FR3	CDR3			
34VL	GNTATLTISKVEAGDEADYYC	QVWSSSDHVV	FGGGTQLTVL		
212VL	-D---L-KS--G-----	-M--HI---MI	---R-K----		

Fig. 4. Amino acid sequences of VH and VL of human anti-VACV mAbs SC34 and SC212.

Complementarity-determining regions (CDR1, CDR2, and CDR3) and framework regions (FR1, FR2, and FR3) are indicated above the sequence alignment. Dashes (-) indicate an identical residue. Asterisks (*) indicate deletion of amino acids.

CHO DG-44 cells were co-transfected with the H and L vectors, selected against geneticin, and growth-adapted up to 1 μ M methotrexate. Cells were then adapted to serum-free suspension culture, and the antibodies were purified using protein A agarose and ion-exchange chromatography.

The binding affinities of VC34 and VC212 were compared by competition ELISA, yielding IC_{50} values estimated at 2 μ g/ml (13.33 nM) and 22 μ g/ml (146.67 nM), respectively (Fig. 5). VC34 exhibited 11-fold higher affinity to VACV as compared with VC212.

Fig. 5. Competition ELISA for measuring relative affinities of anti-VACV mAbs.

Inhibition of anti-VACV mAb binding to VACV-coated plates by free VACV was analyzed using different concentrations of free VACV. Each symbol denotes a clone of VC34 (●) and VC212 (■). Competition ELISA was performed only once due to a lack of VACV availability.

VACV neutralization by anti-VACV mAb

VACV neutralization by anti-VACV mAbs was determined by PRNT. The PRNT₅₀ was defined as the mAb concentration that reduced the number of VACV plaques by 50% as compared with the number of VACV plaques using the culture medium for CHO cells. The PRNT₅₀ of VC212 was estimated at ~0.16 mg/ml (~1.07 μ M) (Fig. 6), whereas VC34 did not exhibit plaque count reduction, but showed an 11-fold higher affinity to VACV relative to VC212.

Discussion

There have been several attempts to screen recombinant VIGs from the phage-display antibody libraries of different genetic sources and antibody formats, such as phage-display chimpanzee Fab libraries [15, 16], the phage-display human Fab library [17], and the phage-display human scFv library [18]. In addition, human mAbs against VACV antigens were screened using KM mice carrying the human chromosome fragments containing the entire human immunoglobulin heavy-chain locus and the YAC transgene for 50% of the human immunoglobulin kappa light-chain locus, and used to select human mAbs against VACV antigens [19].

Chimpanzee Fabs against the VACV B5 envelope protein were isolated from a phage display Fab library derived from the bone marrow of chimpanzees vaccinated with VACV and then converted to mAbs with human IgG1 heavy-chain constant regions (8AH8AL and 8AH7AL with K_d values of 0.2 and 0.7 nM respectively) [15]. The authors demonstrated that mAbs against VACV B5 envelope protein alone protected mice sufficiently from lethal

Fig. 6. Neutralization of VACV by anti-VACV mAb as determined by PRNT.

(A) Plot of the number of plaques *versus* mAb concentration. Each symbol denotes VC34 (●) and VC212 (■). PRNT for VC34 (B) and VC212 (C), with initial concentrations of 2.4 mg/ml and 2.9 mg/ml, respectively, both of which were diluted 16-fold. For PRNT, Vero E6 cells and the VACV_{WR} strain were employed. The PRNT assay was performed only once due to a lack of VACV availability.

challenge with virulent VACV and mice administered 2 days after infection [15]. The efficacy of 8AH8AL and 8AH7AL could not be compared to the efficacy of VC212 due to the absence of PRNT of these mAbs. Human VIG was found to be inferior to chimpanzee-human mAbs in animal studies because of the low concentration of protective antibodies in VIG; 5 mg of VIG contains less than 10 µg of mAb to the B5 envelope protein [15].

Chimpanzee Fabs against the VACV A33 envelope protein were isolated from the same chimpanzee Fab phage library described above, and then converted to mAbs with human IgG1 heavy-chain constant regions (6C, 12C, and 12F with K_d values of 0.14–20 nM) [16]. Two chimpanzee-human mAbs (6C and 12F) showed similar protective efficacy, although 6C exhibited ~140-fold lower affinity for VACV A33 envelop protein than 12F [16]. It was also shown that similar protection was achieved with the mAbs against the VACV A33 envelope protein compared to that with the mAbs against the VACV B5 envelope protein [16].

Human Fabs against VACV were isolated from a phage-display library constructed with lymphocytes of vaccinated donors [17]. The authors collected B cells from vaccinated donors as in the current study, and then constructed the Fab phage library as opposed to the scFv phage library in the current study. They selected 6 Fab clones representing

neutralization of VACV by PRNT. However, they did not show the PRNT with IgG mAbs.

Human scFvs capable of neutralizing VACV were selected from a human scFv phage library by panning against VACV [18]. Construction of the scFv phage library and panning against VACV were very similar to those in the current study; however the authors tested the neutralization of VACV with phage antibodies, which was different from the IgGs that we measured.

Human mAbs against H3 and B5 VACV antigens were produced using KM mice [19]. The authors found that combination therapy with two fully human mAbs against H3 and B5 provided significantly better protection against VACV infection in a small animal model of progressive vaccinia (SCID mice infected with VACV_{NYCBOH} vaccine strain) compared to the use of single human mAbs or VIG. The PRNT₅₀ of human anti-H3 mAb hV26 was estimated to be ~100 ng/ml (~0.67 nM) for neutralization of VACV_{WR} according to the standard overnight neutralization assay. The PRNT₅₀ of VC212 in this investigation against the same VACV_{WR} strain was ~0.16 mg/ml (~1.07 µM), indicating much lower neutralization efficiency of VC212 compared to that of hV26. In addition, the authors showed that in vivo efficacy of 100 µg of human anti-H3 mAb hV26 was similar to that of 1.25 mg VIG. SCID mice treated with a

single dose of 100 µg human anti-H3 mAb hV26 and 1.25 mg VIG at day-1 followed by infection with 1×10^4 PFU VACV_{WR}, mAb hV26 and VIG exhibited similar in vivo protection, as revealed by body weight, % survival, and clinical score [19].

This study solved the disadvantages of human plasma-derived VIG production by isolating human mAbs capable of neutralizing VACV from a phage-display scFv library prepared from B cells of VACV-vaccinated volunteers. As a result, a human anti-VACV mAb VC212 having a PRNT₅₀ of ~0.16 mg/ml (~1.07 µM) was selected. This VC212 provides the basis for further development of VACV-neutralizing human mAbs for overcoming vaccine complications and as agents for prevention of disease after exposure to smallpox.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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