



Evaluation of the fusion partner cell line SPYMEG for obtaining human monoclonal antibodies against influenza B virus

Priyanka SONI¹⁾, Atsuhiko YASUHARA¹⁾, Toru TAKENAGA¹⁾,
Kiyoko IWATSUKI-HORIMOTO¹⁾, Ryuta URAKI¹⁾, Mutsumi ITO¹⁾,
Tadahiro SASAKI²⁾, Kazuyoshi IKUTA²⁾, Seiya YAMAYOSHI¹⁾* and
Yoshihiro KAWAOKA^{1,3-5)}*

¹⁾Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

²⁾Department of Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan

³⁾Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI 53711, U.S.A.

⁴⁾Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

⁵⁾ERATO Infection-Induced Host Responses Project, Japan Science and Technology Agency, Saitama 332-0012, Japan

ABSTRACT. Influenza B virus has been known to infect humans and other animals, including seals. Vaccination efficacy varies across seasons. Human monoclonal antibodies (mAbs) can be useful for developing novel vaccines, guided by epitope analysis, and can be used therapeutically. Hybridoma technology has been used to make mAbs. Here we evaluated SPYMEG as a fusion partner cell line for human mAb generation specific to influenza B hemagglutinin (HA). SPYMEG is a human/murine myeloma partner cell line that has previously been used to generate human mAbs that recognize the HA of influenza A and B viruses. Peripheral blood mononuclear cells were obtained from 16 volunteers, previously vaccinated with the 2014–2015 trivalent seasonal influenza vaccine, and were fused with SPYMEG to yield hybridomas. The resulting hybridomas were screened for antigen-specific antibody secretion and cloned by limiting dilution. We obtained 32 stable clones secreting anti-influenza B HA human IgG, although most of these clones were obtained from one volunteer (SeaV-29) who had a robust immune response. We conclude that SPYMEG is a good fusion partner cell line, although cloning by limiting dilution may lead to significant loss of hybridomas.

KEY WORDS: human monoclonal antibody, hybridoma, influenza B virus, SPYMEG

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Monoclonal antibodies (mAbs) have broad therapeutic, diagnostic, and experimental uses. The technique to generate mAbs, which was first developed in the mid-1970s, involves fusing B-lymphocytes or splenocytes from immunized mice with murine myeloma cells [11] to make antibody-secreting hybridomas. While mAbs generated from mice are commonly used for diagnostics and scientific research today, human mAbs represent a better choice for therapeutics because they are less likely to be immunogenic [17] and result in fast clearance [4]. Several methods exist to generate human mAbs. For example, the hybridoma approach to making human mAbs involves fusing B cells with human or human/murine myeloma cell lines. Peripheral B-cells can also be immortalized by transformation with Epstein-Barr virus [5, 26]. The phage display approach involves expressing the immunoglobulin variable region on bacteriophages, which are then selected for binding to a specific antigen [3, 13]. Recently, high-throughput molecular cloning techniques have made mAb generation possible through the sorting of memory B cells specific for an antigen, followed by a single cell PCR-amplification of the variable regions of the heavy and light chains [12, 27]. Nevertheless, mAb production through hybridoma technology remains one of the most popular methods as evidenced by the number of therapeutics on the market [15].

We sought to generate human mAbs against the influenza B virus surface protein hemagglutinin (HA). Influenza B virus primarily

*Correspondence to: Kawaoka, Y.: yoshihiro.kawaoka@wisc.edu, Yamayoshi, S.: yamayo@ims.u-tokyo.ac.jp

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infects humans and has been known to infect seals [1]. Although research efforts have focused mainly on influenza A virus, which infects a variety of animals such as birds, pigs, and humans [16], influenza B virus continues to be a major cause of morbidity each season, posing a disproportionate health burden on children and the elderly [21, 22]. Neuraminidase inhibitors are commonly used for early treatment, although clinical studies have shown that they are less effective in treating influenza B virus infection compared to influenza A infection [8, 18]. Novel treatments are therefore needed and human mAbs are an attractive option.

For successful mAb generation using hybridomas, choosing the right partner cell line is important. An optimal fusion partner will fuse with high efficiency, will stably grow and maintain antigen-specific monoclonal antibody secretion, and will not secrete antibodies of its own. Fully human cell lines such as U266 and its derived lines were initially used to generate fully human hybridomas, but these cell lines were only modestly successful [25]. Heterohybridoma fusion partners, developed by fusing murine myeloma cell lines with human cells, have been more successful. One such cell line, SHM-D33, has been used to obtain human mAbs against cytomegalovirus [2] and HIV-1 [7]. Another heterohybridoma, K6H6/B5, has been used to create human hybridomas that secrete mAbs to hepatitis C virus [6]. HMMA2.5 has been used to generate mAbs against 1918 H1N1, H1N1pdm2009 [9, 30], and H3N2 [29]. The more recently developed heterohybridoma line SPYMEG is suitable for the production of human mAbs because it does not contain human chromosome deletions for stable heavy and light chain secretion [14], does not secrete human or murine Ig, and is 8-azaguanine-resistant and HAT sensitive [10]. SPYMEG was optimized from a human megakaryoblastic leukemia cell line (MEG-01), obtained from a patient with blast crisis of Philadelphia chromosome-positive chronic myelogenous leukemia, via fusion with SP2 murine myeloma cells [10, 19]. SPYMEG has been successfully used as a fusion partner to obtain mAbs that recognize the HA of influenza A [10, 14, 20] and influenza B viruses [28], and the dengue virus envelope protein [23, 24]. However, as with most partner cell lines, little information regarding the efficiency of SPYMEG has been reported. In this study, we evaluated SPYMEG as a fusion partner to peripheral blood mononuclear cells (PBMCs) from volunteers vaccinated with seasonal influenza vaccine.

MATERIALS AND METHODS

Ethics statement

Human blood was collected according to protocols that were approved by the Research Ethics Review Committee of the Institute of Medical Science, The University of Tokyo, and all experiments were performed in accordance with The University of Tokyo's guidelines and regulations. Written informed consent was obtained from all participants.

Study volunteers and plasma extraction

Sixteen healthy volunteers were recruited for this study, in which they agreed to be vaccinated with the 2014–2015 season trivalent influenza vaccine (TIV). The vaccine was comprised of strains A/California/7/2009 (H1N1pdm09), A/New York/39/2012 (H3N2), and B/Massachusetts/02/2012 (Yamagata-lineage influenza B virus). Volunteer blood was drawn prior to vaccination, and again at 30 and 180 days post-vaccination. Peripheral blood was drawn into a heparin-treated container, and plasma was separated by centrifugation and stored at -20°C before further analysis.

Peripheral blood mononuclear cell (PBMC) extraction and cell fusion

Peripheral blood (20 ml) was drawn into a heparin-treated container one week post-vaccination, and PBMCs were separated with a Ficoll gradient. The PBMCs (8.0×10^5 – 6.0×10^6) were immediately fused with the mouse-derived fusion partner cell line SPYMEG (MBL, Nagoya, Japan) using polyethylene glycol 1500 at a ratio of 10:1. The fused cells were suspended in high-glucose Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS) and hypoxanthine-aminopterin-thymidine (HAT) before being plated into 96-well plates. The cells were then incubated at 37°C in a humidified incubator with 5% CO_2 and monitored daily for colony formation.

Expansion and cloning of hybridomas to produce monoclonal antibodies

Supernatants from wells containing hybridoma colonies were screened for influenza HA-specific antibodies by using an ELISA. Cells positive for HA-specific antibodies were transferred to 24-well plates and maintained in HAT-containing medium. Cells that grew were screened again (secondary screen) and positive wells were cloned by limiting dilution in 96-well plates.

IgG enzyme-linked immunosorbent assay (ELISA)

ELISA plates (96-well) were coated with $2 \mu\text{g}$ (in $50 \mu\text{l}$) of Yam lineage strain B/Florida/4/2006 recombinant HA (Sino Biological Inc., Beijing, P.R. China) overnight at 4°C , and then blocked for 1 hr with $5 \times$ diluted Blocking One (Nacalai Tesque, Kyoto, Japan) at room temperature. After blocking, two-fold serially diluted plasma or cell supernatant was applied for 1 hr at room temperature, after which the wells were washed with Phosphate buffered saline with Tween-20 and IgG was detected by incubation with peroxidase-conjugated goat AffiniPure anti-human Fc γ antibodies (Jackson ImmunoResearch Laboratories Inc., Baltimore, PA, U.S.A.). The wells were developed with 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS), and the reaction was detected at 405 nm. ELISA titers were calculated as the highest dilution with a detection value of ≥ 0.1 .

Virus neutralization test (VNT)

B/Massachusetts/02/2012 was propagated in Madin-Darby canine kidney (MDCK) cells. The MDCK cells were plated in 96-well plates and incubated overnight at 37°C to allow monolayers to form. Plasma was inactivated with Receptor-Destroying

Table 1. Antibody responses in the plasma of volunteers vaccinated with 2014–2015 seasonal vaccine

Volunteer ID	Age	Sex	ELISA titer ($\times 1,000$)			Neutralization titer		
			Pre ^{a)}	Day 30 ^{a)}	Day 180 ^{a)}	Pre	Day 30	Day 180
H5V-2	54	F	8	8	8	32	16	64
H5V-3	26	F	8	8	8	32	256	256
H5V-5	42	M	32	16	32	512	1,024	1,024
H5V-6	30	F	8	8	n/a ^{b)}	128	256	n/a ^{b)}
H5V-8	40	F	4	4	4	32	64	<4
H5V-11	35	M	8	4	4	32	32	16
H5V-19	32	M	64	32	32	1,024	2,048	32
SeaV-21	22	F	16	16	16	32	512	2,048
SeaV-22	24	M	16	4	8	32	64	128
SeaV-23	30	F	16	8	8	128	128	128
SeaV-24	45	F	4	4	8	8	32	128
SeaV-25	34	F	4	8	8	128	128	32
SeaV-28	31	F	8	8	8	128	128	32
SeaV-29	38	F	4	32	8	<4	512	128
SeaV-30	26	F	16	8	n/a ^{b)}	128	512	n/a ^{b)}
SeaV-31	26	F	16	8	n/a ^{b)}	64	64	n/a ^{b)}

a) Plasma samples were taken 7 days prior to vaccination (pre), and on days 30 and 180 post-vaccination. b) Not available.

Enzyme (Denka Seiken Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's instructions and was then serially diluted two-fold in PBS before being incubated with 100 50% Tissue culture infective dose (TCID₅₀) of virus (50 μ l) at 37°C for 30 min. The virus-plasma mixture was then added to the pre-washed MDCK monolayers and left in a humidified incubator for 1 hr at 37°C with 5% CO₂. The virus-plasma mixture was then aspirated and the cells were washed before minimum essential medium (MEM) supplemented with 0.3% bovine serum albumin (BSA) and 1 μ g/ml TPCK-treated trypsin was added. The cells were observed for cytopathic effects (CPE) 72 hr post-infection; neutralization titers were defined as the lowest dilution at which CPE did not occur.

Statistical analysis

The Spearman's rank correlation test was used for statistical comparisons of two data groups. Correlations were considered significant at $P < 0.05$.

RESULTS

Serological titers vary depending on the individual vaccinated

Each volunteer's antibody response to the vaccine was evaluated by using an ELISA and a virus neutralization assay with plasma collected pre-vaccination and on days 30 and 180 post-vaccination. The ELISA revealed that vaccination did not induce a significant antibody response against recombinant HA except in volunteer SeaV-29 (Table 1). In volunteer SeaV-29, the ELISA titer increased at day 30 post-vaccination but dropped down to the pre-vaccination level at day 180 post-vaccination. Virus neutralization titers increased by more than 4-fold in five volunteers (H5V-3, SeaV-21, SeaV-24, SeaV-29 and SeaV-30) at day 30 post-vaccination. Of note, SeaV-29 showed the strongest response to vaccination. The neutralization titers of the volunteers other than these five volunteers were not affected by vaccination.

Hybridomas formed with SPYMEG are not very stable

The fusion of PBMCs isolated from volunteers one week post-vaccination with SPYMEG yielded hybridomas after selection in HAT-containing medium. A large number of wells (2,652/7,680; 34.5%) contained hybridomas. Although all samples yielded hybridomas, the number of hybridomas per sample that varied greatly from 21 to 444 wells did not correlate with the number of PBMCs used for fusion (Table 2). Median number of wells with hybridomas was 148.5. Hybridomas were screened for influenza B HA-specific antibody production by using an ELISA containing commercially available recombinant HA of strain B/Florida/4/2006 as the antigen, which shares 98.8% amino acid identity to the vaccine seed virus B/Massachusetts/2/2012. Because the 2014–2015 trivalent seasonal vaccine did not include a Victoria-lineage strain, hybridomas were only screened against a Yamagata-lineage strain. In the primary screen, although a total of 329 wells were positive for influenza B HA-specific antibodies, 313 were from a single volunteer (SeaV-29) (Table 2). Cloning by limiting dilution reduced well numbers by 65.3% (130/199) and 42.6% (26/61) in the first and second cloning steps, respectively. Ultimately, we obtained 32 clones that stably expressed human mAbs against B-HA.

A robust increase in VNT positively correlates with the number of stable clones obtained

Because we observed an array of antibody responses, we tested whether there was a correlation between a change in VNT or ELISA antibody titer from pre-vaccination to 30 days post-vaccination and the number of stable clones obtained, by using a

Table 2. Production and screening of hybridomas derived from vaccinated volunteers

Volunteer ID	Age	Sex	Number of PBMCs used for fusion	Wells with hybridomas ^{b)}	Primary screen positive wells ^{c)}	Dropped before secondary screen	Secondary screen positive wells ^{c)}	Dropped before 1st cloning step	1st cloning step positive wells ^{d)}	Dropped before 2nd cloning step	2nd cloning step positive wells ^{d)}	Stable clones
H5V-2	54	F	1.3 × 10 ⁶	345/480	0/345	–	–	–	–	–	–	–
H5V-3	26	F	1.5 × 10 ⁶	30/480	0/30	–	–	–	–	–	–	–
H5V-5	42	M	5.0 × 10 ⁶	50/480	0/50	–	–	–	–	–	–	–
H5V-6	30	F	NA ^{a)}	66/480	0/66	–	–	–	–	–	–	–
H5V-8	40	F	NA	21/480	0/21	–	–	–	–	–	–	–
H5V-11	35	M	3.0 × 10 ⁶	187/480	0/187	–	–	–	–	–	–	–
H5V-19	32	M	8.0 × 10 ⁵	204/480	1/204	0/1	0/1	–	–	–	–	–
SeaV-21	22	F	NA	266/480	9/266	0/9	6/9	1/6	1/5	0/1	1/1	1/1
SeaV-22	24	M	1.0 × 10 ⁶	240/480	0/240	–	–	–	–	–	–	–
SeaV-23	30	F	6.0 × 10 ⁶	124/480	0/124	–	–	–	–	–	–	–
SeaV-24	45	F	3.0 × 10 ⁶	151/480	0/151	–	–	–	–	–	–	–
SeaV-25	34	F	1.0 × 10 ⁶	139/480	0/139	–	–	–	–	–	–	–
SeaV-28	31	F	NA	146/480	3/146	1/3	0/2	–	–	–	–	–
SeaV-29	38	F	1.4 × 10 ⁶	444/480	313/444	19/313	225/294	32/225	67/193	8/67	33/59	30/33
SeaV-30	26	F	1.0 × 10 ⁶	56/480	2/56	0/2	2/2	1/2	1/1	0/1	1/1	1/1
SeaV-31	26	F	2.3 × 10 ⁶	183/480	1/183	1/1	–	–	–	–	–	–
Total				2,652/7,680	329/2,652	21/329	233/308	34/233	69/199	8/69	35/61	32/35

a) Not available. b) Wells with cell colonies growing in the presence of HAT-containing medium. Fused cells were plated in five 96-well plates (480 wells). c) Screening by ELISA with recombinant HA of B/Florida/4/2006 (Yamagata-lineage). d) Cells were cloned by limiting dilution and monitored for antibody production by means of an ELISA.

two-tailed non-parametric Spearman rank correlation test. We found that there was a significant relationship between a change in VNT titer and the number of stable clones obtained (Spearman's $\rho=0.65$, $P=0.006$). However, there was no significant relationship between the antibody response measured with the ELISA (Spearman's $\rho=0.27$, $P=0.30$) and the number of stable clones obtained, or between the serologic titers and the ratio of positives to total wells in the primary screen.

DISCUSSION

Here we evaluated the suitability of SPYMEG to serve as a fusion partner cell line for the generation of human mAbs. PBMCs from sixteen volunteers were successfully fused with SPYMEG, and wells containing hybridomas were observed from all samples. Screening for specificity in the primary screen revealed that only six of the sixteen individuals yielded hybridoma-secreting antibodies against influenza B HA (Table 2). Vaccination with seasonal TIV vaccine elicited varying degrees of change in the serologic titers of the immunized volunteers (Table 1). Our evaluation of serologic metrics pre- and post- vaccination and their relationship to the generation of stable clones producing human mAbs revealed a correlation with an increase in VNT from pre-vaccination to 30 days post-vaccination. We therefore recommend screening samples by VNT to increase the likelihood of stable clone generation.

In our study, there was a 90.3% (32 stable clones/329 positive primary wells) drop in antigen-specific hybridomas overall. A large number of cells died off or stopped secreting antigen-specific mAbs during cloning and scaling-up, presumably due to instability of the hybridoma. This is a serious limitation because it not only reduces the total number of mAbs obtained, but also their diversity. Our results show that while the stability of the formed hybridomas is not high, a considerable number of mAbs can be obtained. Therefore, the use of SPYMEG is an option to generate human mAbs against influenza B virus.

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