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ORIGINAL ARTICLE

Successful selection of an infection-protective anti-*Staphylococcus aureus* monoclonal antibody and its protective activity in murine infection models

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ABSTRACT

Recent clinical trials to develop anti-methicillin-resistant *Staphylococcus aureus* (MRSA) therapeutic antibodies have met unsuccessful sequels. To develop more effective antibodies against MRSA infection, a panel of mAbs against *S. aureus* cell wall was generated and then screened for the most protective mAb in mouse infection models. Twenty-two anti-*S. aureus* IgG mAbs were obtained from mice that had been immunized with alkali-processed, deacetylated cell walls of *S. aureus*. One of these mAbs, ZBIA5H, exhibited life-saving effects in mouse models of sepsis caused by community-acquired MRSA strain MW2 and vancomycin-resistant *S. aureus* strain VRS1. It also had a curative effect in a MW2-caused pneumonia model. Curiously, the target of ZBIA5H was considered to be a conformational epitope of either the 1,4-β-linkage between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine or the peptidoglycan per se. Reactivity of ZBIA5H to *S. aureus* whole cells or purified peptidoglycan was weaker than that of most of the other mAbs generated in this study. However, the latter mAbs did not have the protective activities against *S. aureus* that ZBIA5H did. These data indicate that the epitopes that trigger production of high-yield and/or high-affinity antibodies may not be the most suitable epitopes for developing anti-infective antibodies. ZBIA5H or its humanized form may find a future clinical application, and its target epitope may be used for the production of vaccines against *S. aureus* infection.

Key words monoclonal antibody, peptidoglycan, Staphylococcus aureus, vaccine.

Being a major hospital pathogen, wide dissemination of MRSA has posed a serious clinical challenge worldwide (1). Moreover, recent emergence of CA-MRSA, which is known to be more virulent and transmissible than healthcare-associated MRSA, has also become a threat (2, 3). MRSA is a multidrug-resistant pathogen, having generated resistance to almost all the antibiotics so far introduced to clinical use. Recently, it conquered the "last-resort" antibiotic vancomycin by generating vancomycin-intermediate *S. aureus* in 1997 (4, 5) and eventually vancomycin-resistant *S. aureus* (VRSA) in 2002 (6). It is thus evident that MRSA will continue to generate resistance to any antibiotic developed in the future.

In a search for alternative therapeutic strategies for countering MRSA infection, vaccines and protective mAbs have been studied intensively in recent years. These include vaccines against *S. aureus* capsular

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List of Abbreviations: ADCA, alkali-processed, deacetylated cell-wall antigen; CA-MRSA, community-acquired MRSA; GlcNAc, *N*-acetyl-d-glucosamine; (Gly)5, pentaglycine; MRSA, methicillin-resistant *Staphylococcus aureus*; MurNAc, N-acetylmuramic acid; PG, peptidoglycan; TSB, tryptic soy broth; VCM, vancomycin hydrochloride; VRSA, vancomycin-resistant *S. aureus*.

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polysaccharide types 5 and 8 (7, 8) or iron surface determinant B (9, 10). Therapeutic mAbs have also been developed against clumping factor A (11, 12), adenosine triphosphate-binding cassette transporter (13), and teichoic acid (14, 15). However, clinical trials of these vaccines and mAbs have failed to demonstrate sufficient efficacy to allow their introduction into clinical practice (16–18). These facts suggest that the target antigens used thus far are not applicable for the prevention or therapy of *S. aureus* infection.

In this study, we adopted an alternative strategy for obtaining a protective mAb against *S. aureus* infection; namely, immunizing mice with the cell-wall components of *S. aureus* cells. The cell wall components were de-acetylated before immunization to alter their immunogenicity and to obtain a variety of mAbs. The *O*-acetyl moiety of the bacterial polysaccharides has strong immunogenicity, which causes a high proportion of antibodies to be directed against it (19, 20). *S. aureus* has a highly *O*-acetylated PG (21) that is the main component of the cell wall. Therefore, we anticipated that immunization with de-acetylated cell-wall components may help to generate a rare protective mAb against an antigen with a weak immunogenicity. In addition, the de-acetylated component of *S. aureus* biofilms reportedly elicits protective immunity against *S. aureus* infection in mice (22).

Using a panel of 22 mAbs that are reactive against *S. aureus* cell wall components and were obtained by immunization, we screened for mAbs with protective activity in mouse infection models and found one, ZBIA5H, that was protective against *S. aureus* infection in both sepsis and pneumonia models. We report here a curious property of this mAb.

MATERIAL AND METHODS

Bacterial strains and growth conditions

CA-MRSA strain MW2 (23) and VRSA strain VRS1 (6) were obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (Chantilly, VA, USA). *S. aureus* strain OS2 (24) was kindly provided by Olaf Schneewind of the University of Chicago (Chicago, IL, USA). MW2 and OS2 were cultured on TSB or mannitol salt agar at 37 °C. VRS1 was cultured on TSB containing 4 μg/mL VCM (Sigma–Aldrich, St Louis, MO, USA) at 37 °C.

Immunogen preparation

MW2 was cultured on TSB until late logarithmic phase and then collected by centrifugation at 10,000 *g* at 4 °C for 15 min. The cells were lysed using a Bead–Beater homogenizer (BioSpec Products, Bartlesville, OK, USA). The insoluble fraction was collected by centrifugation at 32,000 *g* at 4 °C for 60 min. This pellet was washed three times with 0.2 M phosphate buffer (pH 7.5) containing 1% Triton-X100 (25) and suspended in a 12.5% ammonium hydroxide solution with stirring at 37 °C for 16 hr to yield an ADCA (26). The ADCA was stored at −80°C. An aliquot of 200 mg/mL ADCA was mixed with an equivalent volume of Freund's complete adjuvant or Freund's incomplete adjuvant and emulsified to serve as immunogen.

Immunization

All animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Juntendo University and the Zenyaku Kogyo Research Laboratory.

Ten-week-old female BALB/c mice (Charles River Laboratories Japan, Kanagawa, Japan) were injected intraperitoneally every 2 weeks with 0.2 mL of Freund's complete adjuvant–immunogen (first immunization) or Freund's incomplete adjuvant–immunogen (three subsequent immunizations). Two weeks after the fourth immunization, 5 mg of ADCA was injected into the tail veins of the mice.

Hybridoma production of anti-*S. aureus* antibodies

Three days after the last immunization, cells were isolated from the spleens of immunized mice. The spleen cells were fused with cells of the mouse myeloma cell line, SP2/0-Ag14 (Riken Bioresource Centre, Ibaraki, Japan) using polyethylene glycol (molecular weight 1450; Sigma–Aldrich), after which the hybridomas were single-cell cloned as previously described (27).

Purification of mAbs

Seven- to nine-week-old male mice with severe combined-immunodeficiency (CLEA Japan, Tokyo, Japan) were injected intraperitoneally with 0.5 mL of pristane (Sigma–Aldrich). Two weeks later, the mice were injected intraperitoneally with 5×10^6 hybridoma cells. One to two weeks later, the mice were killed by exsanguination under anesthesia and ascitic fluid collected and centrifuged at 1900 *g* at 4 °C for 10 min. The supernatants were collected and stored at −70°C until required. The antibodies were purified from the ascites supernatants by affinity chromatography using HiTrap Protein-G Sepharose (GE Healthcare, Waukesha, WI, USA), according to the manufacturer's instructions. The purified antibodies were concentrated

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to 5 mg/mL in Dulbecco's PBS, using a Centricon plus or Amicon ultrafiltration unit (EMD Millipore, Billerica, MA, USA), and clarified by passing through a 0.45 μm filter before storage at −70°C.

Indirect whole-cell ELISA

The OS2 strain, which is a protein A-deficient mutant strain, was used in whole-cell ELISAs to reduce non-specific binding to mAbs. OS2 cells were cultured in TSB for 16 hr and collected by centrifugation. The cells were washed three times with PBS and the OD of the bacterial cell suspension at 600 nm was adjusted to 0.1 in PBS. Aliquots (100 μL) of this bacterial suspension were added to each well of a Nunc Maxisorp 96-well ELISA plate (Thermo Scientific, Waltham, MA, USA), and then incubated at 4 °C for 6 hr. After washing three times with PBS, the wells were blocked with 300 μL of 1% rabbit serum in PBS at 4 °C for 16 hr. After washing three times with PBST, 100 μL of antiserum or hybridoma culture supernatant was added to each well and the plates incubated at 30 °C for 2 hr. After washing three times with PBST, 100 μL of an anti-mouse IgG, IgM (Invitrogen, Carlsbad, CA, USA), or anti-mouse IgG(γ) (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) $F(ab')_2$–HRP-conjugate was added to each well and the plates incubated at 30 °C for 2 hr. After washing three times with PBST, 100 μL of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) substrate was added to each well and the absorbance measured at 405 nm (A_{405}), using a microplate reader, to quantify the bound antibody in each well.

Indirect peptidoglycan ELISA

An indirect PG ELISA was performed using 96-well ELISA plates, as described previously (28). PG from *S. aureus* (Sigma–Aldrich) was ultrasonicated for 2 min at 20 kHz and diluted in PBS to 50 μg/mL. The wells were coated with 150 μL of the sonicated PG solution at 4 °C overnight. After washing three times with PBST, 100 μL of the mAbs, diluted in PBST, was added to each well and the plates incubated at 37 °C for 2 hr. After three washes with PBST, 50 μL of dilute (1 μg/mL) goat anti-mouse IgG(γ) $F(ab')_2$–HRP conjugate was added and the plates incubated for 1 hr at 37 °C. Antigen-binding was colorimetrically measured, as described in the "Indirect whole-cell ELISA" section.

Competitive indirect peptidoglycan ELISAs

Competitive ELISAs were performed to determine the PG epitope targeted by each mAb. The following solutions of PG components (all purchased from Sigma–Aldrich) were prepared in PBST: 10 mM GlcNAc, 10 mM MurNAc, 10 mM (Gly), and 4.5 mM Ala-D-γ-Glu-Lys-D-Ala-D-Ala. Solutions of enzymatically digested PG were prepared by incubating PG (1.5 mg/mL) with 0.1 mg/mL lysostaphin (Wako Pure Chemical, Osaka, Japan) or 0.1 mg/mL mutanolysin (Sigma–Aldrich) for 20 hr at 37 °C. Lysostaphin cleaves the pentaglycine component of PG, whereas mutanolysin cleaves the 1,4-β-linkages between the MurNAc and GlcNAc components of PG. The samples were subsequently boiled for 10 min to inactivate the enzyme and then diluted to 0.2 mg/mL in PBST.

The plates, coating conditions and detection method used for the competitive ELISAs were identical to those described in the "Indirect peptidoglycan ELISA" section. After washing the antigen-coated wells three times with PBST, 25 μL of ZBIA1H, ZBIA2H, ZBIA5H or ZBIA6H antibodies and 75 μL of a peptidoglycan competitor or undigested PG were added to each well, after which the plates were incubated at 37 °C for 2 hr. The affinity of each mAb for the PG component and enzymatically digested PG were estimated from the differences in ELISA responses for each antibody in the presence and absence of the added PG component, or in the presence of added enzymatically digested PG or undigested PG.

Indirect lipoteichoic-acid ELISA

An indirect lipoteichoic-acid ELISA was performed using 96-well ELISA plates, as previously described (29). The wells were coated with 100 μL of 2 μg/mL *S. aureus* lipoteichoic acid (Sigma–Aldrich) in PBS and the plates incubated at 37 °C for 16 hr. The mAbs were diluted in PBST containing 2% BSA. After washing the wells three times with PBST, 100 μL of dilute mAb was added to each well and the plates incubated at 37 °C for 1 hr. After washing three times with PBST, 100 μL of goat anti-mouse IgG(γ) $F(ab')_2$–HRP conjugate, diluted in PBST–BSA, was added to each well, followed by incubation at 37 °C for 1 hr. Antigen-binding was then colorimetrically measured, as described in the "Indirect whole-cell ELISA" section.

In vivo lethal sepsis model

Seven-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) were challenged intraperitoneally with a 0.5 mL injection containing approximately 6×10^8 CFU of MW2 or approximately 3×10^9 CFU of VRS1 from late-logarithmic-phase cultures; this was followed by a 0.2 mL intraperitoneal injection of 1 mg of anti-*S. aureus* mAb, 1 mg of normal mouse IgG (EMD Millipore) or 1 mg of VCM (Sigma–Aldrich). The amounts of injected reagents essentially corresponded

to the dosage of VCM for humans (40 mg/kg/day). The animals were monitored and their mortality recorded.

In vivo pneumonia model

Seven-week-old female BALB/c mice were infected by intranasal inhalation of 40 μL of approximately 5×10^8 CFU of MW2 from a late-logarithmic-phase culture (day 0). On day 3, the mice received a 0.2 mL i.v. injection containing 1 mg of ZBIA5H, 1 mg of normal mouse IgG or 1 mg of VCM. On day 5, the mice were killed by exsanguination under anesthesia. The lungs were removed and homogenized in saline solution. Aliquots were then diluted and plated on an agar medium to determine the titer of MW2 in the lungs.

Statistical analysis

The statistical significance of differences in the lethal sepsis data between the various treatment groups was determined using the log–rank test. The statistical significance of differences in the pneumonia data between the various treatment groups was determined using the Kruskal–Wallis and Steel–Dwass tests.

RESULTS

The target of binding of anti-*S. aureus* mAbs

After immunization of mice with the cell-wall components of *S. aureus*, hybridomas were produced and whole-cell ELISA was used to screen their culture supernatants for their ability to bind to *S. aureus* cells . Anti-*S. aureus* antibodies were detected in 106 of 1002 wells. The hybridoma cells from 30 of these wells with an $A_{405} > 2$ were single-cell cloned by limiting dilution. In this way, 22 hybridoma cell lines that produced anti-*S. aureus* IgG mAbs were generated.

The 22 mAbs showed varying degrees of binding affinities to *S. aureus* cells (Fig. 1a). Among them, 16 mAbs bound to *S. aureus* PG (Fig. 1b), five mAbs bound to lipoteichoic acid (Fig. 1c) and one mAb (ZBIA18H) showed a weak affinity to both PG and lipoteichoic acid (Fig. 1b and c). The concentrations of the mAbs that provided A_{405} of 1 in Figure 1 are shown in Table 1.

The effects of mAbs in an *in vivo* lethal sepsis model

Among the 22 hybridomas, three were low-producer cell lines. The other 19 mAbs were tested for their abilities to prevent infection in a mouse lethal sepsis model with

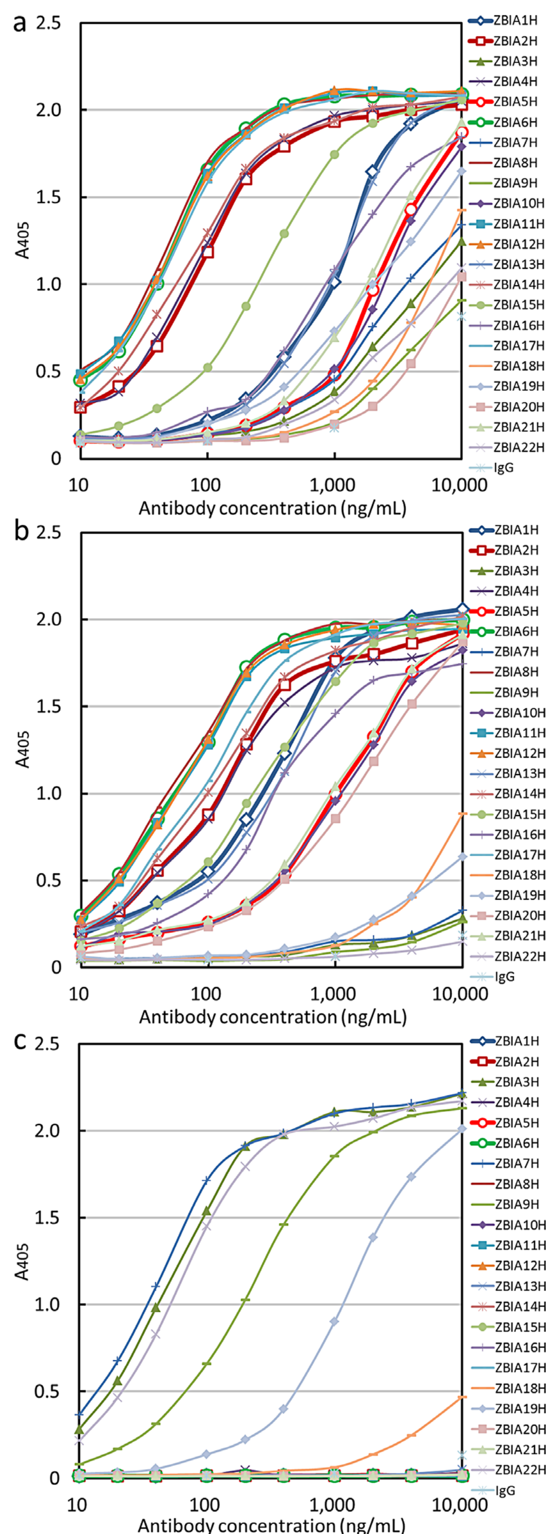

Fig. 1. Binding curves of anti-*Staphylococcus aureus* monoclonal IgG antibodies in (a) indirect *S. aureus* whole-cell ELISA, (b) indirect peptidoglycan ELISA and (c) indirect lipoteichoic-acid ELISA. Each point represents the antibody concentration and its absorbance at 405 nm.

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Table 1. Concentrations of the anti-*Staphylococcus aureus* mAb that provided A_{405} of 1 in *S. aureus* whole cell (cell-), peptidoglycan (PG-) and lipoteichoic acid (LTA)-ELISA (ng/mL).

	ELISA		
mAb	Cell	PG	LTA
ZBIA1H	1000	290	10,000<
ZBIA2H	80	140	10,000<
ZBIA3H	6000	10,000<	40
ZBIA4H	70	140	10,000<
ZBIA5H	2400	1,000	10,000<
ZBIA6H	28	60	10,000<
ZBIA7H	4000	10,000<	35
ZBIA8H	26	50	10,000<
ZBIA9H	10,000<	10,000<	200
ZBIA10H	2600	1200	10,000<
ZBIA11H	28	60	10,000<
ZBIA12H	28	60	10,000<
ZBIA13H	1000	340	10,000<
ZBIA14H	60	100	10,000<
ZBIA15H	260	230	10,000<
ZBIA16H	900	360	10,000<
ZBIA17H	30	90	10,000<
ZBIA18H	6000	10,000<	10,000<
ZBIA19H	2200	10,000<	1250
ZBIA20H	10,000	1,500	10,000<
ZBIA21H	2000	900	10,000<
ZBIA22H	8000	10,000<	50

MW2 as the causative agent (Table 2). In the lethal sepsis experiments, an anti-PG mAb ZBIA5H (IgG2b) was found to have the greatest protective activity of all the mAbs. Although the anti-lipoteichoic acid mAbs ZBIA3H and ZBIA9H were implicated as having protective activity, only the results of our research on ZBIA5H are presented here. ZBIA5H was selected because it has previously been suggested that another anti-lipoteichoic acid mAb (pagibaximab), which has similar targets to ZBIA3H and ZBIA9H, has anti-infection efficacy (14, 15); however, this was not confirmed in clinical trials (17, 18). The course of infection is illustrated in Figure 2a: half of the animals in the ZBIA5H-treatment group were still alive by the fifth day of MW2 infection, whereas 90% of the animals in the normal IgG-treatment groups died within 2 days after infection ($P < 0.05$).

Next, the protective activity of ZBIA5H was tested in a sepsis experiment using VRSA strain VRS1. On the fifth day of VRS1 infection, the survival rate of the ZBIA5H-treated group was 67%, whereas that of the VCM treatment and IgG treatment groups was 44% and 22%, respectively (Fig. 2b; overall $P < 0.05$). Thus, ZBIA5H improved the survival rate in mouse models of infection induced by both CA-MRSA and VRSA strains.

The effects of ZBIA5H in an *in vivo* pneumonia model

The *S. aureus* CA-MRSA strain MW2 used in this study is known to cause necrotic pneumonia (2). Therefore, we evaluated the effect of ZBIA5H against MW2 using a mouse pneumonia model.

On day 2 post-treatment, the median MW2 titer was 8.4×10^6 CFU per lung in the normal IgG-treated groups. In contrast, the median titer in the ZBIA5H-treated group was 1.3×10^4 CFU per lung (Fig. 3). Thus ZBIA5H treatment significantly reduced the titers of MW2 in mouse lungs ($P < 0.01$ by the Steel–Dwass test for ZBIA5H vs. IgG, and overall $P < 0.01$ by the Kruskal–Wallis test). The median MW2 titer in the VCM treatment group was 3.2×10^5 CFU per lung ($P < 0.12$ by the Steel–Dwass test for VCM vs. IgG). Because the half-life of ZBIA5H is assumed to be approximately 4 to 6 days in mice, in this regime involving a single injection of the reagent, ZBIA5H might be more effective than VCM in inhibiting the proliferation of MW2 in the lungs of infected mice.

Determination of the epitope recognized by anti-peptidoglycan mAbs

ZBIA5H binds to *S. aureus* PG (Fig. 1b, Table 1). To further identify the epitopes recognized by the anti-PG

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Table 2. Effects of the anti-*S. aureus* mAb on MW2-induced sepsis, evaluated for 5 days post-infection. The statistical significance (*P*-value) of the differences in the lethal sepsis data between the mAb and control IgG treatment groups was determined using the log-rank test.

	First exp. screening ($n = 4$)	Second exp. screening ($n = 6$)	First + second exp. screening ($n = 10$)
mAb	*P*	*P*	*P*
ZBIA1H	1.000		
ZBIA2H	0.317		
ZBIA3H	0.006	1.000	0.063
ZBIA4H	1.000		
ZBIA5H	0.127	0.181	0.038
ZBIA6H	1.000		
ZBIA7H	NT		
ZBIA8H	0.544		
ZBIA9H	0.040	0.102	0.047
ZBIA10H	0.317		
ZBIA11H	0.317		
ZBIA12H	0.317		
ZBIA13H	0.544		
ZBIA14H	0.127	0.129†	0.567†
ZBIA15H	0.317		
ZBIA16H	NT		
ZBIA17H	0.153	0.613	0.148
ZBIA18H	0.317		
ZBIA19H	NT		
ZBIA20H	0.317		
ZBIA21H	0.040	0.597	0.282
ZBIA22H	0.098	0.197†	0.750†

NT, not tested.
†survival rate was less than in IgG treated groups.

mAbs, competition ELISA experiment was performed with four defined molecular components of PG; GlcNAc, MurNAc, $(Gly)_5$ and pentapeptide Ala-D-γ-Glu-Lys-D-Ala-D-Ala. Three representative PG-binding mAbs ZBIA1H (low affinity for *S. aureus* PG), ZBIA2H (medium-high affinity for *S. aureus* PG), and ZBIA6H (high affinity for *S. aureus* PG) were tested together with ZBIA5H for comparison (Fig. 4). Binding of ZBIA1H to solid-phase PG was inhibited by addition of $(Gly)_5$ (Fig. 4). Hence, the target epitope of the antibody is likely

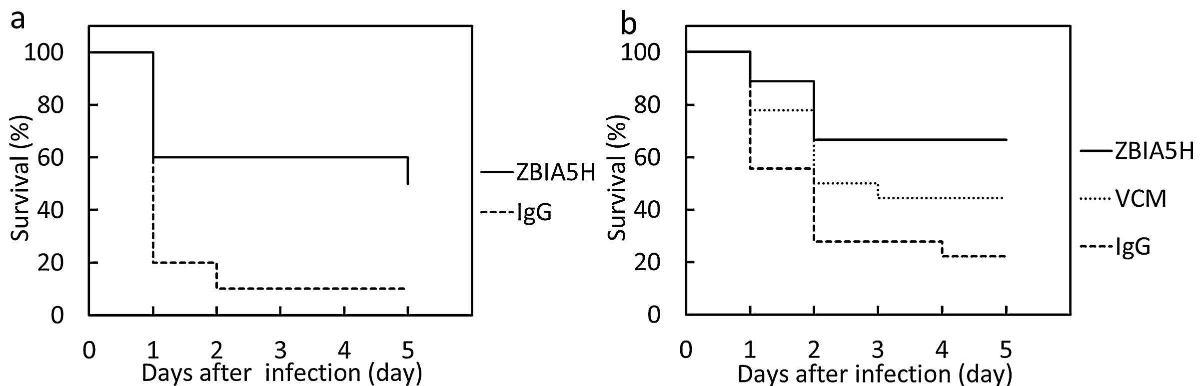

Fig. 2. Effects of ZBIA5H in a mouse model of lethal sepsis using the *Staphylococcus aureus* strains (a) MW2 and (b) VRS1. Seven-week-old female BALB/c mice (a, $n = 10$ per group; b, $n = 18$ per group) were challenged intraperitoneally with *S. aureus* MW2 (5×10^8 to 7×10^8 CFU) or VRS1 (3×10^9 to 4×10^9 CFU) from a late-logarithmic-phase culture, and subsequently injected intraperitoneally with 1 mg of ZBIA5H, 1 mg of normal mouse IgG or 1 mg of VCM. The animals were monitored and the course of infection was observed. The percentage of surviving mice in each treatment group is shown ([a] $P < 0.05$ and overall [b] $P < 0.05$ by the log–rank test).

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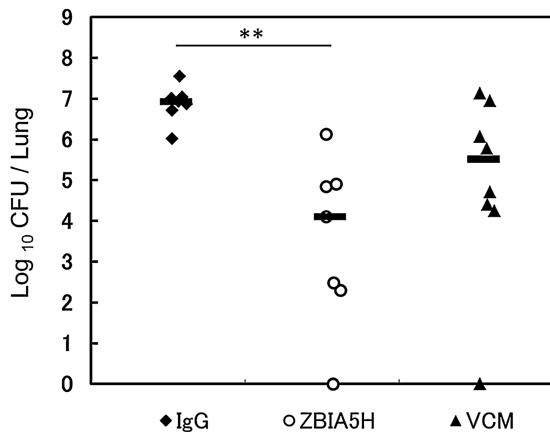

Fig. 3. **Treatment effects of ZBIA5H in a mouse model of pneumonia using the *Staphylococcus aureus* strain MW2.** Seven-week-old female BALB/c mice were infected by intranasal inhalation of MW2 (40 μL; 3×10^8 to 7×10^8 CFU) from a late-logarithmic-phase culture (day 0). On day 3, the mice received 1 mg of ZBIA5H, 1 mg of normal mouse IgG or 1 mg of VCM i.v. On day 5, the mice were killed and their lungs removed and homogenized in saline solution. Aliquots were diluted and plated on an agar medium to determine MW2 titers. Data are shown as the bacterial count (CFU) observed in the lungs of individual animals (ZBIA5H, $n = 7$; control IgG, $n = 7$; VCM, $n = 8$). The horizontal line indicates the median. The detection limit was 100 CFU per lung (overall $P < 0.01$ by the Kruskal–Wallis test; $^{**}P < 0.01$ for ZBIA5H-treated vs. IgG by the Steel–Dwass test).

to be present in $(Gly)_5$. In addition, binding of ZBIA2H and ZBIA6H to PG was inhibited by addition of MurNAc, indicating that their binding epitopes contain MurNAc. In contrast, the reactivity of ZBIA5H, remained almost unchanged in the presence of any of the four components (Fig. 4). Therefore the binding epitope of ZBIA5H was not confined to any of the four components of the PG building block.

In an going search for the epitope of ZBIA5H, the affinity of ZBIA5H to enzymatically-digested PG was next measured by competition ELISA, using native PG or enzymatically-digested PG as the competitor (Fig. 5). Native PG and lysostaphin-digested PG equally inhibited the PG ELISA reactivity of ZBIA5H (Fig. 5a). However, mutanolysin-digested PG did not inhibit the binding of ZBIA5H to solid-phase PG at all (Fig. 5b). These results strongly indicate that mutanolysin-digestion disrupts the epitope to which ZBIA5H binds.

DISCUSSION

Several mAbs targeting the virulence-associated antigens of *S. aureus* have been developed in the past; however, to date none of them have been introduced successfully into clinical practice. In this study, instead of raising mAbs against the well-known surface determinants, we prepared a panel of mAbs against the cell-wall components of *S. aureus*. We then screened these mAbs for their protective effects against *S. aureus* infection in mouse infection models. We this identified an anti-PG mAb, ZBIA5H, that demonstrated good protective activity in a mouse model of sepsis induced by the CA-MRSA strain MW2 and the VRSA strain VRS1, as well as better growth-inhibitory activity than a single injection of VCM against MW2 in a murine lung pneumonia model.

Since its discovery in the 1950 s, VCM has been widely used as the first choice antibiotic for MRSA infection (30, 31). However, recent emergence of vancomycin-intermediate *S. aureus* (4, 5) and VRSA (6) may be a prelude to an ominous scenario in which multidrug-resistant MRSA strains against which no extant antibiotics are effective are prevalent world-wide. It would be wise to prepare for the future by developing as many new anti-MRSA therapeutics as possible. Raising humanized mAb protective against *S. aureus* infection is a plausible approach. However, approaches targeting several defined epitopes have not been successful in the past. In this study, we tried a different strategy for identifying effective mAbs and pinpointed ZBIA5H, which showed better activity than VCM in VRSA-induced sepsis and CA-MRSA-induced pneumonia in mice. Curiously the epitope recognized by ZBIA5H is on the PG, but not confined to any of the four components of its building block, a murein monomer. Interestingly, the epitope was disrupted by mutanolysin treatment. These findings suggest that the epitope of ZBIA5H is either the 1,4-β-

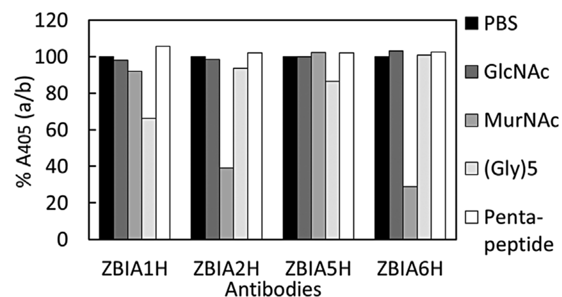

Fig. 4. **Inhibition of mAb-binding to PG by PG components.** Solutions containing 10 mM GlcNAc, 10 mM MurNAc, 10 mM $(Gly)_5$ and 4.5 mM Ala-D-γ-Glu-Lys-D-Ala-D-Ala (pentapeptide) were prepared in PBST. To each well of the peptidoglycan-coated ELISA plate, 25 μL of ZBIA1H, ZBIA2H, ZBIA5H or ZBIA6H (final concentration, 1 μg/mL) and 75 μL of a competitor were added and the plates incubated at 37 °C for 2 hr. Data are shown as the ratio of a/b where a is the A_{405} of competitor and each antibody addition and b is the A_{405} of control PBS and each antibody addition.

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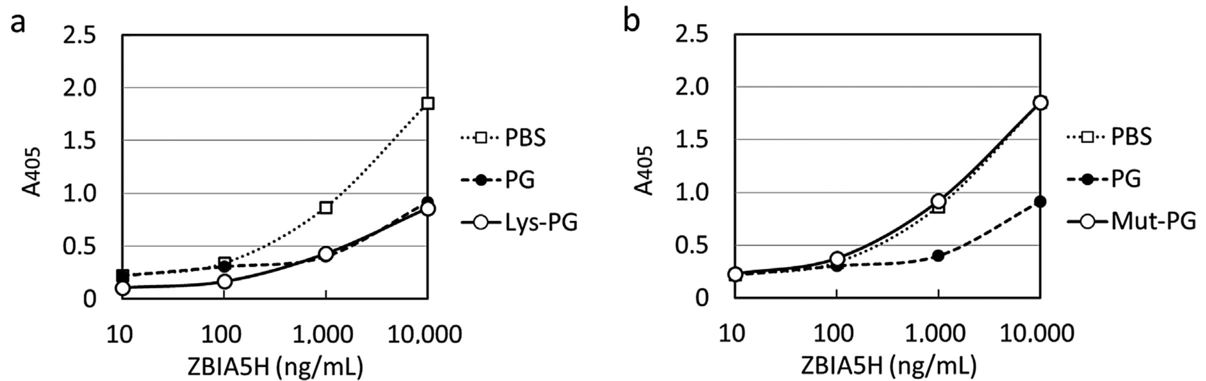

Fig. 5. Binding curves of ZBIA5H in competitive indirect peptidoglycan ELISAs using enzymatically digested PG as a competitor. Monoclonal antibodies ZBIA5H (25 μL) and 75 μL (0.2 mg/mL) of a PG competitor (a) PBS, undigested PG or lysostaphin digested PG. (b) PBS, undigested PG or mutanolysin digested PG were added to each well and incubated at 37 °C for 2 hr. Each point represents the ZBIA5H concentration and its absorbance at 405 nm. Lys-PG, lysostaphin digested PG; Mut-PG, mutanolysin digested PG.

linkages between MurNAc and GlcNAc or that formed by the three-dimensional structure of PG.

Peptidoglycan is the main component of the cell wall of gram-positive bacteria and it is known that titers of anti-PG antibodies increase in patients infected with *S. aureus* (32). Moreover, although vaccines based on the peptide mimics of PG (33) and *S. aureus* cell-wall components that have been purified using concanavalin A-agarose (34) have demonstrated protective effects against *S. aureus* infection in mice, successful protection against infection by an anti-PG mAb has not yet been reported.

Among the 16 anti-PG mAbs generated in this study, ZBIA5H exhibited the best activity in animal infection models. However, its binding affinity to PG was not particularly high compared with the other 15 mAbs (Fig. 1b, Table 1). Interestingly, most of the mAbs, such as ZBIA1H (affinity for $[Gly]_5$), ZBIA2H (affinity for MurNAc), or ZBIA6H (affinity for MurNAc), which showed higher affinity for PG than ZBIA5H, were ineffective in the *in vivo* infection experiments. Previously, candidate protective antibodies against *S. aureus* have been selected based on their greater binding affinities to specific components of *S. aureus* (35). However, our results suggested that the infection-protective activity of an antibody does not always depend on its affinity for the cell component. The reason is unknown, but the ZBIA5H epitope may have weak antigenicity. If this is the case, the ZBIA5H epitope would make a good vaccine candidate. Alternatively, the binding of ZBIA5H may modulate *S. aureus* cell physiology through an as yet unidentified signaling pathway and attenuate its virulence. This possibility is under investigation.

The majority of antibodies raised in this study did not possess effective protective activities. Effective anti-infective antibodies are rare. Antibodies may need to be directed to a limited number of epitopes on *S. aureus* cells or to those with low antigenicity. Moreover, the epitopes that trigger high-yield or high-affinity antibodies may not be suitable targets for the development of anti-infective antibodies. This may explain why no efficient protective antibody against *S. aureus* infection has been developed successfully to date, and why it is difficult to acquire effective immune memory against *S. aureus* infection (36).

In summary, we generated 22 anti-*S. aureus* mAbs by immunizing mice with alkali-processed, deacetylated *S. aureus* cell walls. Among these, we identified an infection-protective anti-PG mAb, ZBIA5H, by using mouse infection models. ZBIA5H recognizes a novel epitope that is vulnerable to mutanolysin digestion. ZBIA5H or its humanized form may find a useful application in prevention and therapy of *S. aureus* infection.

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DISCLOSURE

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HO and JE are employees of Zenyaku Kogyo. TB and KH have no conflicts of interest to declare.

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