97. Kaiser J. Clinical trials. Gene transfer an unlikely contributor to patient's death. Science 2007;318:1535

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Characterization of human single-chain antibodies against highly pathogenic avian influenza H5N1 viruses: mimotope and neutralizing activity

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The development of new therapeutic targets and strategies to control highly pathogenic avian influenza (HPAI) H5N1 virus infection in humans is urgently needed. Neutralizing recombinant human antibodies would provide important agents for immunotherapy on human H5N1 virus infection and definition of the critical mimotope for vaccine development. In this study, we have characterized an anti-H5-specific scFv clone, 3D1 from the human-scFv-displaying phage library. 3D1 blocked the binding of H5-Fc to MDCK cells in flow cytometry and neutralized H5N1 subtype influenza A viruses in a microneutralization assay. Employing a peptide-displaying phage library, Ph.D-12, the mimotope was determined to be at #128-131 and #204-211 of H5, which are silic acid-binding regions. In consistency with this result, 3D1 binds the recombinant sugar-binding domain (#50G-#272E) produced by a baculovirus vector. The 3D1 antibody employs the germline gene VH1-23. As this antibody is the first human anti-H5 scFv clearly defined on the sugar-binding epitope, it allows us to investigate the influence of amino acid substitutions in this region on the determination of the binding specificity to either sialic acid α2,6-galactose (SA α2,6Gal) or sialic acid α2,3-galactose (SA α2,3Gal) providing new insight for the development of effective H5N1 pandemic vaccines.

Keywords: HA/mimotope/neutralizing activity/phage library/scFv.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FITC, fluorescent isothiocyanate; H5-Fc, H5 HA fused with immunoglobulin Fc; HPAI, highly pathogenic avian influenza; HRP, horseradish peroxidase; mAb, monoclonal antibody; MDCK, madin darby canine kidney; MOI, multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PE, phycocerythrin; rHA, recombinant haemagglutinin; rSBD, recombinant sugar-binding domain; scFv, single chain Fv; $TCID_{50}$, median tissue culture infectious doses; TU, transforming unit; VH, variable domain of immunoglobulin heavy chain; VL, variable domain of immunoglobulin light chain.

Influenza viruses continue to cause problems globally in human and their livestock, particularly poultry and pigs, as a consequence of antigenic drift and shift, resulting frequently and unpredictably in novel mutant and re-assortant strains, some of which acquire the ability to cross species barriers and become pathogenic in their new hosts (*1*). Prospects for the emergence of pandemic strains of swine and avian origin have been discussed in several recent reports (*2*, *3*). Human disease due to the direct transmission of the highly pathogenic avian influenza (HPAI) virus of the subtype H5N1 from poultry was first reported in 1997 and resulted in the death of 6 of the 18 infected individuals (*4*, *5*). Re-emergence of HPAI-H5N1 viruses occurred in 2003 and continues to be a cause of disease in both humans and poultry (*6*). Currently, HPAI-H5N1 subtype strains do not transmit efficiently from person to person, a trait that has probably limited the spread to the human population, and most human cases remain a result of a direct bird-to-human transmission (*7*). Nearly 400 human HPAI-H5N1 virus infections have been reported since 1997 from 14 countries; with a case mortality rate in the immunocompetent population ~60% (*8*), there is great concern that a pandemic caused by this influenza virus would result in extremely high morbidity and have major economic consequences (*9*).

Currently, there are a number of obstacles for the development of safe and effective pandemic influenza vaccines (*10*, *11*). Surviving patients who have recovered from H5N1 infection may have neutralizing antibodies, thus suggesting that antibody-mediated immunity may contribute to eliminating infection (*12*). The advantages of human antibodies, particularly their lack of immunogenicity, make them very attractive for therapeutic applications. Fully human monoclonal antibodies (mAbs) with neutralizing H5N1

activity were generated from Epstein Barr virus (EBV)-immortalized memory B cells of infected donors (*13*) or genetically engineered and transiently expressed in mammalian recipient host cells (*14*, *15*) These findings highlight the potential of immunotherapy as a viable treatment option in human cases of avian influenza. However, influenza viruses rapidly mutate, particularly in the regions of haemagglutinin (HA) responsible for antigenicity (*16*), suggesting that the development of a useful passive immunization strategy should include simple and rapid procedures to promptly respond to the threat of an influenza pandemic.

Phage antibody technology appears to offer an option, as human antibody fragments can be isolated from repertoires of fragments displayed on filamentous bacteriophage (*17*, *18*). Recently, we constructed a large scale of the human scFv-display phage library. This library was constructed using only complete human V genes amplified from the IgM and IgG cDNA clones of human peripheral blood. It is a salient feature that these products have been tolerated by the human immune system and are immunologically the safest for humans.

Thus far, human anti-H5 antibodies and scFvs with cross-clade reactivities have been reported, although their binding epitopes have not been determined. Anti-H5 antibodies reactive to either the conserved region of HA2 or a conserved pocket in the stem region of H5 HA have also been reported.

In this article, we report the establishment of human scFv specific to the sugar-binding domain of H5. The binding mimotope was defined by biopanning employing a recombinant domain of the sugar-binding region and epitope mapping using a random-peptide-displaying phage library. Thus, this antibody provides a novel for us to investigate the influence of amino acid substitutions in this region on the determination of the binding specificity to either sialic acid α2,6-galactose (SA α2,6Gal) or sialic acid α2,3-galactose (SA α2,3Gal). Furthermore, this antibody may be useful for passive immunization against H5N1 virus infection.

Materials and Methods

Viruses and cells

The influenza virus strains were A/Vietnam/1194/2004 (H5N1). All infectious material was handled in a bio-safety level-2 or -3 facility under approved protocols in accordance with the guidelines of Hokkaido University or Kagoshima University. These viruses were concentrated and purified by high-speed centrifugation of infected allantoic fluid passed through a 10–50% sucrose density gradient (*19*). The purified viruses were resuspended in phosphate-buffered saline (PBS) and stored at −80°C until use. Madin–Darby canine kidney (MDCK) cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum.

Recombinant full-length HA

The recombinant HA (rHA) from the virus strain A/Vietnam/1194/2004 (H5N1) fused with immunoglobulin Fc (H5-Fc) and the H5-Fc fusion protein is expressed using recombinant baculoviruses and was purified from the infected cell supernatant by Protein A chromatography; it was purchased from Aalto Bio Reagents, Ltd. (Dublin, Ireland).

Preparation of recombinant baculovirus and expression of sugar-binding domain protein in insect cells

The sugar-binding domain of A/Vietnam/1194/2004 H5N1 isolates containing a 0.669 kb DNA fragment of the gene (amino acid 50–272) was chemically synthesized through the Hokkaido System Science Co., Ltd. (Hokkaido, Japan). This region contains 130-loop, 220-loop and 190-helix responsible for the receptor-binding site. A recombinant baculovirus carrying the recombinant sugar-binding domain (rSBD) gene was generated using a baculovirus expression vector system (baculovirus vector: pAcGP67-A) according to the manufacturer's protocol.

Spodoptera frugiperda (Sf9) cells were infected with a recombinant baculovirus at a multiplicity of infection of 0.5. The H5 rSBD soluble protein was purified from the suspension by a His-tag column (GE Healthcare). Finally, the purified H5 rSBD protein was pooled, concentrated and dialyzed against PBS. Aliquots of the purified protein were stored at −20°C until use. Protein concentrations were estimated by their absorption at 280 nm using a molar absorption coefficient calculated by the formula: $\varepsilon M = \text{Trp} \times 5500 + \text{Tyr} \times 1490 + \text{Cystine} \times 125$ as described in ref. (*20*).

Phage library

The human scFv-displaying M13 phage library was constructed using the pCANTAB 5E (4.5 kb) phagemid vector as described earlier (*21*). The scFv gene segments were constructed by ligating the VH gene derived from the μ-chain cDNA with either Vκ-cDNA (μ/κ-library) or Vλ-cDNA (μ/λ-library) or the VH gene derived from the γ-chain cDNA with either Vκ-cDNA (γ/κ-library) or Vλ-cDNA (γ/λ-library). The μ-library was composed of a μ/κ-library and a μ/λ-library at 5×10^{11} transforming units (TU) each, and the γ-library was a combination of the γ/κ-library and the γ/λ-library at 5×10^{11} TU each. The peptide-displaying phage libraries (Ph.D-12) were purchased from New England Biolabs (Beverly, MA, USA).

Biopanning

Biopanning was performed as described earlier (*22*, *23*). Briefly, an Immunotube (Nunc, Nagoya) was coated with recombinant H5-Fc at 6 μg/ml (1× PBS pH 7.1) for panning at 4°C overnight. The H5-Fc-coated tube was blocked with 0.5% gelation for first-round selection and 0.25% BSA for second-round selection. A library (5×10^{11} TU) consisting of VH-Vλ (λ-library) or VH-Vk (k-library) was incubated in an H5-Fc-coated tube at room temperature for 1 h. The tube was washed 10 times with PBS containing 0.1% Tween-20 (PBST). The bound phages were eluted with 1 ml 0.1M glycine–HCl (pH 2.2) and immediately neutralized with a 0.1 volume of 1 M Tris–HCl (pH 9.1). Phage amplification or preparation was performed as described in ref. (*21*). In the case of the peptide-phage library, biopanning was performed as described earlier (*21*). Briefly, an immunotube (Nunc) was coated with 3D1 scFv at 5 μg/ml in 0.1M $NaHCO_3$ (pH 8.6). The isolated phage clones were amplified by infecting into *Escherichia coli* ER2738 cells.

Soluble scFv

The soluble scFv was prepared by infecting phage clones with *E. coli* HB2151 (a non-suppressor strain) as described in ref. (*21*). These supernatant or periplasmic scFvs were purified with an anti-E-tag affinity column (RPAS purification module, GE Healthcare Bio-science Corp., Piscataway, NJ, USA). Shimadzu LC20 high-performance liquid chromatography (HPLC, Shimadzu, Kyoto) was performed as follows: scFvs were loaded on a 10/300 GL Superdex75 (GE Healthcare Bioscience Corp., Piscataway), which was developed in PBS at a flow rate of 0.5 ml/min. The monomer form of scFv was fractionated. The anti-E-tag affinity column (GE Healthcare Bio-science Corp., Piscataway) was discontinued. Therefore, the original C-terminal tag (E-tag) of scFv was replaced with a His-tag by recombination of the gene from pCANTAB5E to a pCANTA5H vector to generate scFv/pCANTA5H phage. The phage was infected to *E. coli* HB2151, and the scFv–His-tag was expressed in HB2151 by induction of 1 mM IPTG at 30°C. The bacterial cells were treated by 1 mM EDTA/PBS, and the supernatant obtained by centrifugation was supplied to affinity purification on a His Trap^{TM}HP column (GE Healthcare), according to the manufacturer's instructions.

ELISA

ELISA was performed as described in (*24*, *25*). Briefly, antigens (50 ng/40 µl/well) were adsorbed to a microtiter plate (Nunc, Denmark). Phage clones [40 µl of PEG-precipitated phage (10^{12} TU)] or soluble scFv (100 ng/40 µl/well) were added to the wells. Phage clones were detected by a biotinylated anti-M13 mAb (1 : 1000, Pharmacia, CA, USA) in combination with AP-conjugated streptavidin, and absorbance was measured at 405 nm by the use of a microplate reader. The soluble scFv antibody was detected using HRP-conjugated anti-E-tag mAb (Pharmacia) or mouse anti-His-tag mAb in combination with HRP-conjugated goat anti-mouse IgG (H+L) (Jackson Immuno Research, West Grove, PA, USA) at a dilution of 1 : 1000. Absorbance was measured at 450 nm by the use of a microplate reader (NJ-2300; Nunc, Tokyo).

Immunoblotting analysis

The periplasmic and supernatant fraction of the HB2151 culture was subjected to SDS–PAGE (12.5%) and blotted to a PVDF membrane using a semidry electroblotter. After blocking with 5% skimmed milk, it was detected by horseradish peroxidase (HRP)-conjugated anti-E-tag mAb (Pharmacia) using ECL reagents (GE Healthcare Bio-science Corp., Piscataway) on an image analyzer LAS-1000 (Fujifilm, Tokyo). The NEB pre-stained protein marker (New England Biolabs, Beverly, MA, USA) was used as the protein mark.

DNA sequencing

The nucleotide sequence of the scFv genes was identified using the Dye Terminator Cycle Sequencing FS Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) with primer 1 (5′-CAACGTGAAAAAATTATTATTCGC-3′) for the scFv gene or primer 2 (5′-CCCTCATAGTTAGCGTAACG-3′) for the peptide insert. The amino acid residues of each variable domain were according to Kabat and colleagues (*26*, *27*).

Influenza virus strain ELISA

ELISA was performed essentially as described earlier (*17*). Briefly, purified viruses were disrupted with 50 mM Tris–HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl, diluted by PBS, and used for antigen coating (20 µg protein/ml in PBS, 50 µl/well), followed by blocking with BSA. Binding of the scFv antibody was detected by using mouse anti-His-tag mAb in combination with peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) (Jackson Immuno Research, West Grove). Mouse monoclonal IgG antibody 61_2_1 against the H5-VN04 virus was detected using HRP-conjugated goat anti-mouse IgG (H+L) at a dilution of 1 : 1000. Absorbance was measured at 450 nm by the use of a microplate reader.

Flow cytometric analysis

Flow cytometry analysis was performed as described earlier (*22*). Briefly, the mammalian MDCK (Madin Darby canine kidney) cells with 80–90% confluent growth were collected by centrifugation and washed in cold PBS and FACS buffer (PBS containing 10% FBS and 0.1% sodium azide). The MDCK cells (1×10^6 cells) were stained with H5-Fc (10 µg/ml) followed by the addition of phycoerythrin (PE)-conjugated F(ab′) Fragment of affinity Purified anti-Human IgG (Sigma, St Louis, MO, USA) and analyzed with EPICS flow cytometer (Beckman-Coulter). To examine the inhibitory activity of 3D1 scFv, H5-Fc was pre-incubated with scFvs at 4°C for 1 h before the flow cytometry analysis. To determine the binding activity of rSBD to MDCK cells, MDCK cells were incubated with rSBD on ice for 1 h. rSBD binding was monitored with mouse anti-His-tag mAb and FITC-anti mouse IgG mAb (Jackson Immuno Research, West Grove) with the EPICS flow cytometer (Beckman-Coulter).

Neutralization assay

The method was performed as described earlier (*28*). Briefly, 100 $TCID_{50}$ (median tissue culture infectious doses) of the virus (A/Vietnam/1194/2004) were mixed in equal volumes with 2-fold serial dilutions of an scFv Ab stock solution (50 µg/ml) in 96-well tissue culture plates and incubated for 2 h at 37°C. Indicator MDCK cells (1.5×10^4 cells per well) were added to the plates, followed by incubation at 37°C for 18 h. To establish the endpoint, cell monolayers were then washed with PBS and fixed in acetone (80%), and a viral antigen was detected by ELISA with a mAb against influenza A NP (A-3, Accurate) and HRP-conjugated goat anti-mouse IgG (H+L) (Jackson Immuno Research, West Grove).

Epitope analysis

This assay was performed to determine the 3D1 epitope by selecting 3D1-specific peptide phage clones. Briefly, an ELISA plate was coated with 3D1 scFv (100 ng/50 µl/well) for 12 h at 4°C. Peptide phage clones (5.0×10^{10} virions/50 µl/well) were added to each well for 1 h. The phage clones were detected by a biotinylated anti-M13 mAb in combination with AP-conjugated streptavidin.

Results

Identification of anti-H5N1 HA scFv clones by ELISA

The current H5N1 epidemics involve viruses derived from a single lineage of H5 HA. Within this lineage, four distinct clades have been identified as major threats to public health (*29*, *30*). H5-Fc-specific scFv-phage clones were selected from the scFv-phage libraries. As we intended to select phage clones with varying affinities, two and three rounds of biopanning were carried out. The 241 clones were tested by ELISA. We selected 27 clones that showed fine specificity to H5-Fc (data not shown). Of those, 3D1 phage clones that yielded positive signals greater than the background by ELISA were selected for further studies. Those phage clones did not bind to control proteins, including BSA and human IgG, indicating that the phage-scFv antibodies are specific for the antigen, H5-Fc (Fig. 1).

The soluble scFvs were induced by IPTG after each phage clone was infected into *E. coli* HB2151. The periplasmic and supernatant fraction of each clone was electrophoresed in an SDS–polyacrylamide gel (12.5%) under reducing conditions and immunoblotted with an HRP-conjugated anti-E-tag mAb. This analysis showed that the 3D1 scFv was expressed in both the periplasmic and supernatant fraction. 3D1 was resolved at ~27–28 kDa (Fig. 2a). The 3D1 scFv was purified using anti-E-tag affinity column with an elution buffer of 0.1 M glycine–HCl (pH 3.0). In agreement with these results, gel permeation chromatography showed that the 3D1 scFv was resolved in a monomer form (Fig. 2b).

Reactivity of soluble scFv to HA was tested by ELISA using several H5-Fc and the influenza virus

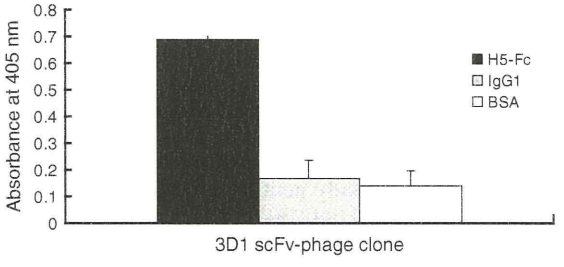

Fig. 1 H5-binding activity of scFv-phage clones. ELISA was performed using plates coated with H5-Fc, human IgG or BSA (50 ng/40 µl/well) in the presence of each phage clone (8×10^{10} virions/40 µl/well). The binding of scFv-phage clones to antigen was detected by a biotinylated anti-M13 mAb (1 : 1000, Pharmacia) in combination with AP-conjugated streptavidin. Human IgG and BSA were used as negative controls. ELISA was performed as described in 'Materials and Methods' section.

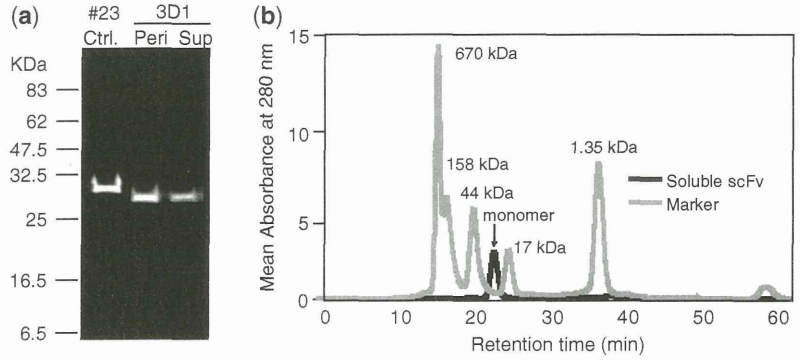

Fig. 2 Characterization of soluble scFv. (a) SDS–PAGE; the periplasm (peri) and supernatant (sup) of 3D1 pahge-infected HB2151 cell culture were subjected to 12.5% gel SDS–PAGE under reducing conditions. After electroblotting, the scFvs were detected with the anti-E-tag monoclonal antibody. (b) Gel-permeation chromatography (10/300 GL Superdex75) of the 3D1 scFv antibody.

H5N1 strain. Purified soluble scFvs showed fine binding specificity to H5-Fc (Fig. 3a), but no binding to control proteins (BSA, human IgG). The soluble 3D1 scFv antibody also showed binding specificity to the influenza virus H5-VN04 strain in a dose-dependent manner, although the 3D1 scFv antibody showed lower binding activities than the positive control mouse monoclonal IgG antibody 61_2_1 against the H5-VN04 virus and the unrelated scFv (Su1) showed no binding to the H5-VN04 strain (Fig. 3b). The 3D1 scFv gene sequences of the selected phage clones were examined (Fig. 4a and b). The germ-line VH and VL genes were assigned on the basis of homology to a database (IMGT) of germ-line V genes compiled by Lefranc *et al.* (*23*, *31*).

Inhibition of H5N1 HA binding to MDCK cells by scFv antibody

3D1 inhibits the binding of H5-Fc to MDCK cells, and MDCK cells were incubated with 3D1 in the presence of varying concentrations of scFvs. H5-Fc binding was monitored with PE-conjugated anti-human IgG using an EPICS flow cytometer. As shown in Fig. 5, 3D1 inhibited the binding of H5-Fc to MDCK cells in a dose-dependent manner and showed ~70% inhibition at 25 μg/ml of scFvs. The unrelated #23 scFvs had no influence on the binding of H5-Fc to MDCK cells.

Neutralization assay

The neutralizing activity of 3D1 was determined by a microneutralization assay using MDCK cells (Fig. 6). Consistent with its reactivity profile in an FACS assay, the 3D1 scFv antibody neutralized infectivity of virus strain, A/Vietnam/1194/2004. However, the 3D1 scFv antibody showed lower neutralizing activities than positive control mouse monoclonal IgG antibody 61_2_1 against virus strain, A/Vietnam/1194/2004. These results indicated that the 3D1 antibody had a potential for practical therapy against the infectivity of influenza A viruses by inhibiting HA binding to the sialic acid receptors, as it is still a single chain antibody and, furthermore, one of human origin.

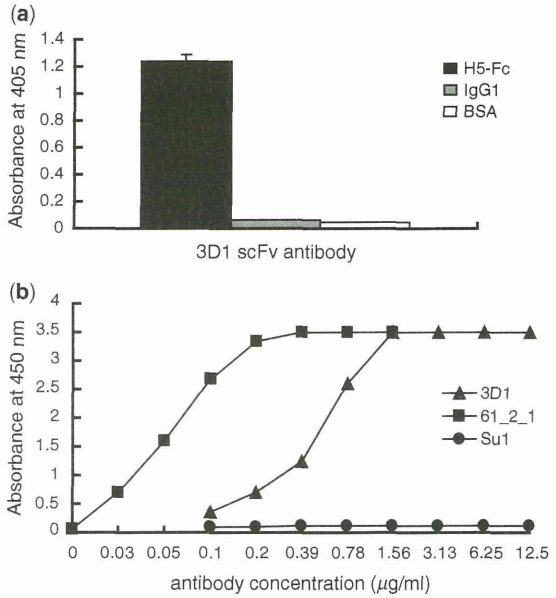

Fig. 3 H5-binding activity of soluble scFvs. (a) ELISA was performed using plates coated with H5-Fc, human IgG or BSA (50 ng/40 μl/well) in the presence of purified 3D1 scFv (100 ng/40 μl/well) as described in 'Materials and Methods' section. (b) Reactivity of 3D1 scFv Ab to the influenza virus strain (H5-VN04) with indicated concentrations. Su1 was used as unrelated scFv antibody; 61_2_1 was used as positive control antibody. ELISA was performed as described in 'Materials and Methods' section.

3D1 scFv binds to the rSBD of H5 HA

rSBD prepared by a baculovirus expression vector system was analysed by SDS–PAGE (Fig. 7a). The binding activity of rSBD to MDCK cells was determined by flow cytometry analysis (Fig. 7b). The results indicated that rSBD bound MDCK cells in a dose-response manner although it was relatively weak. This might be because the form of rSBD used was monovalent due to the absence of a foldon region in this construct. To examine whether rSBD was correctly folded to function, we tested the binding of rSBD to 3D1 scFv by ELISA. As His-tag-column-fractionated

(a) Amino acid sequence of the VH domain

FLR1 CDR1 FLR2 CDR2
QVQLQQSGGGLVQPGGSLRLSCAASGFTFS **DLAMT** WVRQAPGKGLEWVS **SISGSGDNTYYADSVKG**
FLR3 CDR3 FLR4
RFTISRDNSKNTLYLQMNSLRAEDTALYYCAN **EGAFDI** WGQGTTVTVSS

(b) Amino acid sequence of the VL domain

FLR1 CDR1 FLR2 CDR2
EIVLTQSPGTLSLSPGERATLSC **RASQSVSSSYLA** WYQQKPGQAPRLLIY **GASTRAT**
FLR3 CDR3 FLR4
GIPVRFSGIGSGTDFTLTISRLEPEDFAVYYC **QQYGSSPPT** FGPGTKVDIKR

(c) Variable gene usage

	Heavy chain			Light chain	
clone	V	D	J	V	J
3D1	IGHV3-23*01. or IGHV3-23*04	n. d	IGHJ3*02 (a)	IGKV3-20*01	IGKJ3*01

Fig. 4 The amino acid sequence of the 3D1 scFv clone deduced from the DNA sequence. The complementary determining regions (CDR1–CDR3) and the frame regions (FR1–4) were assigned according to Broxhet *et al* (*31*). VH domains (a) and VL domains (b). CDR regions are indicated in bold. The scFv nucleotide sequences were analysed by searching the IMGT/V-QUEST database to identify the gene usage on the immunoglobulin germ line (c) (*31*). The D regions of VH chain were not determined for assignment of corresponding gene segments.

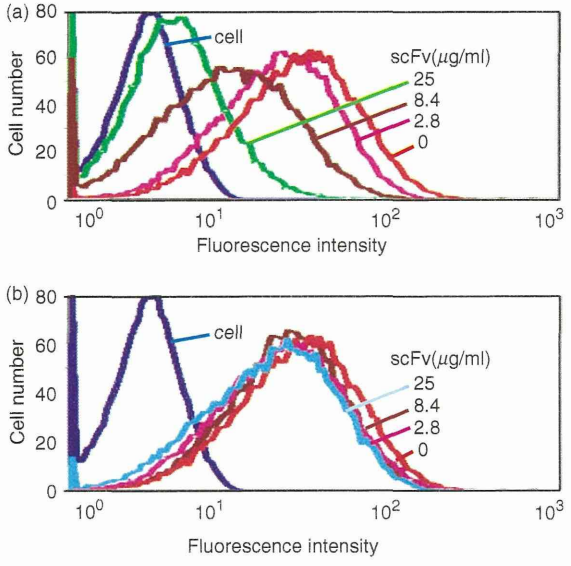

Fig. 5 The 3D1 scFv inhibits the binding of H5-Fc to MDCK cells. MDCK cells (1×10^6 cells/ml) were incubated with H5-Fc (10 μg/ml), which had been pre-incubated with varying concentration of scFv for 60 min at 4°C. H5-Fc-binding MDCK cells were stained with PE-conjugated anti-human IgG Abs and analysed by flow cytometry. (a) 3D1; (b) #23 scFv; Ab: unrelated scFv.

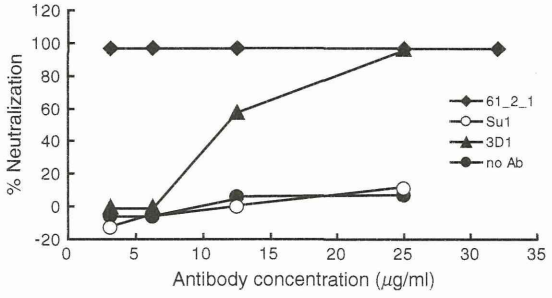

Fig. 6 Neutralizing activity of the 3D1 antibodie against A/Vietnam/1194/2004 (H5N1). Neutralization assay was performed as described earlier (*28*). MDCK cells were infected with H5N1 influenza virus pre-incubated with serial dilutions of antibodies. Eighteen hours later, MDCK cells were fixed with 80% aceton and stained with anti-influenza A NP antibody in conjunction with HRP-conjugated goat anti mouse IgG antibodies (*28*). The percentage of neutralization was defined by the following formulas: Background A_{450} (MDCK alone) was subtracted from each experimental A_{450} value. {[(A_{450} of MDCK with virus—A_{450} of MDCK with virus pre-incubated with 3D1 scFv)/A_{450} of MDCK with virus] × 100%. Irrelevant scFv antibody (Su1) or murine monoclonal IgG antibody 61_2_1 specific to A/Vietnam/1194/2004 (H5N1) virus were used as a negative or positive control antibody, respectively.

preparation of rSBD contained irrelevant proteins that was detectable by silber staining, ELISA was performed using plastic plates coated with varying concentrations of highly purified 3D1scFv, followed by the addition of His-tag-fractionated rSBD. As shown in Fig. 7c. rSBD showed binding specificity to the 3D1 in a does-dependent manner, but not to an unrelated scFv antibody (#23), indicating the structural homogeneity of this rSBD preparation.

Epitope mapping of 3D1 scFv

The 3D1 mimotope clones were selected from Ph.D-12 peptide-displaying phage libraries (Ph.D-12 for linear peptides) using 3D1 scFv antibody-coated ELISA microtitre plates. Twenty clones (~80% of selected clones) were tested for the binding specificity. Six clones specifically bound to 3D1 scFv (Fig. 8). DNA sequencing analysis showed that five of six clones bore an identical motif (pep3D1-1: LPSSSPTPF LSG). One had a unique sequence (pep3D1-9: GTT MPLHAAPHS). Homology search using CLUSTAL W version 3.1 have indicated that there are two weakly homologous regions splitted at #204-211 and

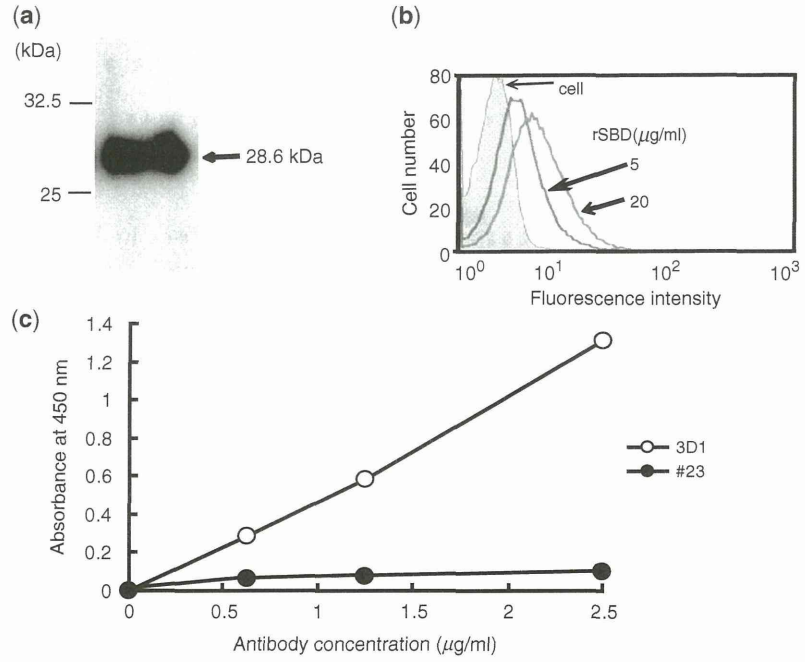

Fig. 7 H5 sugar-binding domain (H5 rSBD) recognized with 3D1 scFv. (a) SDS–PAGE analysis of His-tag-colum-fractionated H5 rSBD protein. The H5 rSBD protein was electrophoresed in 12.5% gel under reducing conditions. The H5 rSBD protein was blotted onto a PVDF membrane, blocked with 5% skim milk, and probed with anti-His-tag mAb. (b) Flow cytometry analysis was performed with MDCK cells. Cells were incubated with rSBD at the concentrations of 5 and 20 μg/ml. The concentration was estimated as described earlier (*20*). rSBD binding was monitored with mouse anti-His-tag mAb and FITC-anti mouse IgG mAb. (c) rSBD-binding activity of 3D1 scFv by ELISA. Microtiter plates were pre-coated with a serial concentration of scFv antibody, ranging from 2.5 to 0.8 μg/ml, followed by the addition of 3 μg/ml of rSBD. The binding of H5 rSBD to scFv antibody was detected by using mouse anti-His-tag mAb and HRP-conjugated anti mouse IgG mAb. ELISA was performed as described in Fig. 3.

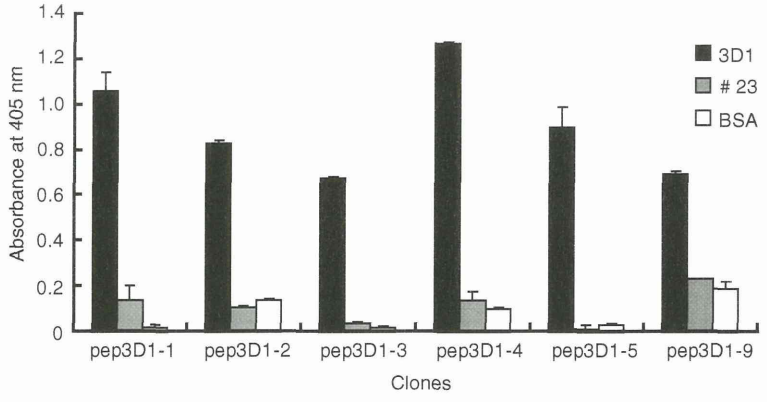

Fig. 8 Epitope analysis: Ph.D-12 clones specifically bound to 3D1 scFv: 3D1 scFv-specific peptide-displaying phage clones were isolated from peptide-displaying phage library (Ph.D-12). The ELISA plate was coated with 3D1 or #23 scFv (100 ng/50 μl). The phage clones (5.0×10^{10} virions/50 μl) were added to the wells, and their binding was detected by a biotinylated anti-M13 mAb in combination with AP-conjugated streptavidin.

#128-131 in the case of pep3D1-1) while pep3D1-9 sequence motif showed weak homology at the regions splitted at #287-293 and #367-368 of H5 HA molecule of A/Vietnam/1194/2004 influenza virus strain (Fig. 9a). The H5 HA structure was depicted according to the PyMoL (molecular visualization system) using a database of the PDB code, 2IBX, as shown in Fig. 9b. These regions correspond to the 190-helix and 130-loop of H5 HA, which have been reported as regions responsible for binding sites to sialic acid α2, 6-galactose (SA α2,6Gal) or sialic acid α2, 3-galactose (SA α2,3Gal) (*32*).

Discussion

In the present study, we established a human anti-H5 scFv, 3D1, by biopanning with recombinant H5-Fc from a naïve scFv-displaying phage library.

(a)

Virus or clone name	Amino acid sequence	Frequence
	121 131 141 151 161 171 181 191 201 211	
H5 (VN1194)	HFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPKDAAEQTKLYQNPTTYISVGTS	
pep3D1-1	- - - -LPSS- SPTPFLSG- -	5/6
	:*.* .**. ::*	

(b)

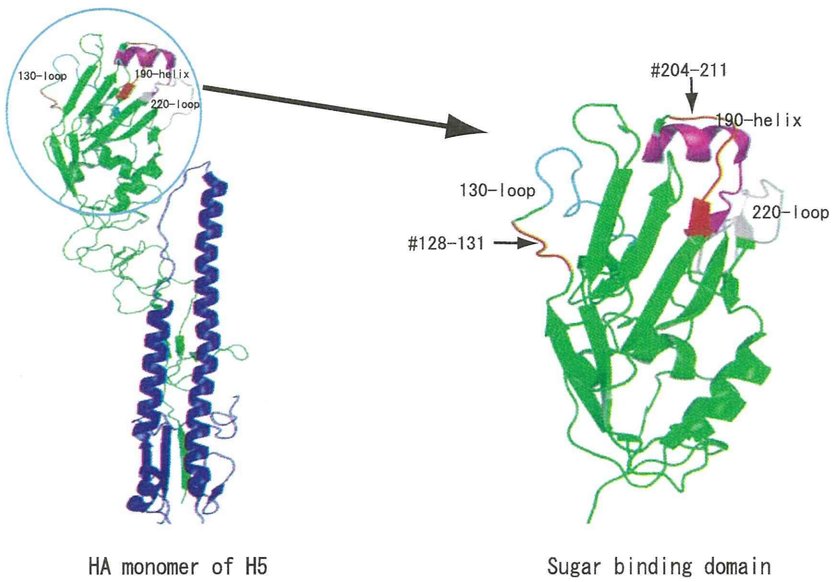

Fig. 9 Major epitope of 3D1 scFv locates at the H5 sugar-binding region. (a) A major epitope of 3D1 scFv, pep3D1-1 is analysed on the homologous region with H5 HA using CLUSTAL W ver. 3.1. The mark, 'asterisk', 'colon' or 'dot', indicated below the epitope sequence, indicates identical, conserved or semi-conserved residues, respectively. The weakly homologous regions correspond to the 190-helix and 130-loop of H5 HA. (b) Magnified picture of the globular head of H5. The H5 hemagglutinin structure was depicted according to the PyMoL (molecular visualization system) using a database of the PDB code, 2IBX. The binding site comprises three structure elements: an 1-helix (190-helix) and 2-loops (130-loop and 220-loop), which have been reported as regions responsible for binding sites to sialic acid α2,6-galactose (SA α2,6Gal) or sialic acid α2,3-galactose (SA α2,3Gal) (*32*). The epitope regions of 3D1 scFv are depicted in red (#204-211 and #128-131).

3D1 showed a neutralizing activity (Fig. 6) even if it was scFv and was purified as a monovalent form by gel permeation chromatography (Fig. 2). To investigate the binding epitope of 3D1, the specific binding clones to 3D1 from a peptide-display phage library (Ph.D-12) were selected, and their binding peptide sequences were determined. Homology analysis of CLUSTAL W showed that the major epitope sequences were weakly homologous to #204-211 and #128-131 of H5 HA of the A/Vietnam/1194/2004 influenza virus strain. These regions correspond to the 190-helix and 130-loop of H5 HA, which have been reported as the regions responsible for binding sites to sialic acid α2,6-galactose (SA α2,6Gal) or sialic acid α2,3-galactose (SA α2,3Gal) from comparative analyses of the amino acid sequences of varying H5N1 isolates (A/Vietnam/1194/2004) (*32*). This finding suggests that 3D1 might recognize the conformation structure but not the linear epitope of the sugar-binding domain of H5. We demonstrated a consistent result in which 3D1 bound to the rSBD (#50G-#272E) produced by the baculovirus vector system.

Although human anti-H5 scFvs or Fab antibodies with cross-clade reactivities have been reported (*28*, *33–35*), their binding epitopes have not been determined. Anti-H5 antibodies reactive to either the conserved region of HA2 or the conserved pocket in the stem region of H5 HA have also been reported.

However, as 3D1 is the first human scFv of which the mimotope has been defined on the sugar-binding epitope, it is possible to identify the amino acids contributing the fine binding specificity in this region by amino-acid substitution experiments. Furthermore, 3D1 antibody may be useful for passive immunization against H5N1 virus infection.

Conflict of interest

None declared.

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