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Catalytic and Biochemical Features of a Monoclonal Antibody Heavy Chain, JN1-2, Raised against a Synthetic Peptide with a Hemagglutinin Molecule of Influenza Virus

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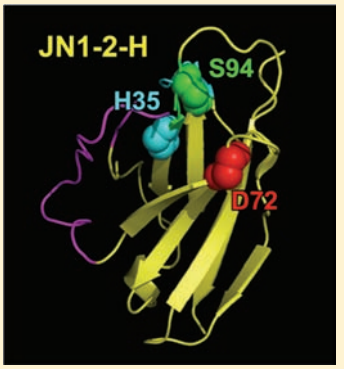
ABSTRACT: It has long been an important issue to produce a catalytic antibody that possesses the ability to lose the infectivity of a bacteria or virus. The monoclonal antibody JN1-2 was generated using a synthetic peptide (TGLRNGITNKVNSVIEKAA) conjugated with human IgG. The peptide sequence includes the conserved region of the hemagglutinin molecule (HA_1 and HA_2 domains), which locates on the envelope of the influenza virus and plays an important role in influenza A virus infection. The monoclonal antibody specifically reacted with the HA2 domain, not only of H2 but also of an H1 strain of the H1N1 subtype (H1 strain). The heavy chain (JN1-2-H) isolated from the parent antibody showed catalytic activity cleaving the above antigenic peptide with very high turnover (kcat = 26 min^{-1}), and it could slowly degrade the recombinant HA_2 domain by the catalytic function. Interestingly, the heavy chain exhibited the ability to reduce the infectivity of type A H1N1 but not type B, indicating specificity to type A. This characteristic monoclonal catalytic antibody heavy chain could suppress the infection of the influenza virus in vitro assays.



INTRODUCTION

One of the potential utilities of catalytic antibodies is for therapeutics, especially against infectious agents, through the specific destruction of essential proteins in a virus or bacteria. Many catalytic antibodies degrading antigens such as VIP,[1] DNA,[2] HIV gp41,[3] HIV gp120,[4] factor VIII,[5] etc. have been reported, in the past decade.

From the standpoint of a new approach to generate or characterize a catalytic antibody, Gololobov et al. reported a unique catalytic antibody cleaving gp120 of HIV, using a covalently reactive analogue (CRA) method.[6] Ponomarenko et al. made a catalytic anti-idiotypic antibody and examined its features in detail using several potential peptide substrates.[7] A new concept of a catalytic vaccine using catalytic antibodies was realized by Gabibov et al.[8] In addition, Hifumi et al. reported a unique characteristic antibody light chain capable of suppressing the number of *Helicobacter pylori* infecting mice stomach.[9] Because many unique catalytic antibodies have been developed to date, their application as therapeutic agents will become a reality in the near future.

In recent years, the pandemic possibility of worldwide influenza virus infection has become a crucial issue. Influenza A virus can be subtyped according to the serological reactivities of its surface antigens, hemagglutinin (HA), and neuraminidase (NA) molecules. There are sixteen HA subtypes (H1∼H16) and nine NA subtypes (N1∼N9).[10,11] As a result, many subtypes of the influenza A virus could exist such as H1N1 (H1 strain), H2N2 (H2 strain), H3N2 (H3 strain), H5N1 (H5 strain), etc. The former three strains (H1, H2, and H3), which caused worldwide pandemics, originated in Europe in 1918, in Asia in 1957, and in Hong Kong in 1968, respectively.[12,13] The latter strain (H5) is recognized as an avian influenza virus. One of the most important steps required for the influenza virus to infect human cells is that the hemagglutinin molecule on the surface of the virus binds and fuses with the membranes of human cells. Therefore, the hemagglutinin molecule (HA) is essential in the infection. In this study, we attempted to prepare a catalytic antibody capable of specifically destroying the hemagglutinin molecule for the purpose of preventing infection.

As stated above, there are 16 (H 1∼H16) subtypes of hemagglutinin molecules (HA) in the influenza A virus. Okuno et al.[14] reported the highly conserved sequences of the hemagglutinin molecule present among the influenza A virus (H1N1 and H2N2). One sequence (TGLRN) was present in the HA_1 domain of hemagglutinin and another (GITNKVNSVIEK) in the HA_2 domain. In this study, both sequences were chemically synthesized as a polypeptide (TGLRNGITNKVNSVIEK) by combining them. By injecting BALB/c mice with the synthetic peptide conjugate with h-IgG, we succeeded in preparing a catalytic antibody heavy chain (antigenase) capable of cleaving the injected antigenic polypeptide. In addition, it could suppress

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the infectivity of the influenza virus. This paper presents the details of the features of the heavy chain (JN1-2-H) and the results of an in vitro assay.

METHODS

A polypeptide, TGLRNGITNKVNSVIEK, was synthesized by the Fmoc method as an Ala-Ala-Cys adduct formed at the C-terminal and conjugated with human IgG (h-IgG) using the Cys residue for the immunogen. The conjugate was subcutaneously injected with Freund's adjuvant, and the spleen cells were fused with myeloma cells using polyethylene glycol followed by HAT selection, screening, and cloning.[15−17] In accordance with ref 18, the heavy chain (JN1-2-H) was isolated and purified from the parent mAb by reducing the antibody with 2-mercaptoethanol, blocking with iodoacetamide, purification by size-exclusion chromatography, and then dialysis for refolding.

Before the degradation reaction of the antigenic peptide by JN1-2-H was conducted, most glassware, plasticware, and buffer solutions were sterilized by heating (180 °C, 2 h), autoclaving (121 °C, 20 min), or passing through a 0.2 μm sterilized filter. Manipulations in the experiment were mostly performed in a safety cabinet to avoid airborne contamination.

The degradation reaction was conducted in a 15 mM phosphate buffer (pH 6.5) at 25 °C. For monitoring of the reaction, 20 μL of the reacting solution was injected into an RP-HPLC system (Jasco) under isocratic conditions (0.05% trifluoroacetic acid and 12.5% acetonitrile) with a column temperature of 40 °C. A degradation assay for the influenza virus was monitored by SDS−PAGE followed by Western blot analysis.

Computational analysis of the antibody structures was performed using the deduced VL and VH amino acid sequences with a workstation (Silicon Graphics Inc., PA, USA) running AbM software (Oxford Molecular Ltd., Oxford, UK), which is used for building models of three-dimensional molecules.

Synthesis of an Antigen Peptide. We adducted two alanine molecules at the C-terminal portion as TGLRNGITNKVNSVIEKAA to hold the helix conformation, which was synthesized by the Fmoc method (Funakoshi Co. Ltd., Tokyo). Regarding conjugation with h-IgG, another peptide adducted with cysteine at the C-terminal was prepared and then conjugated with h-IgG using the cysteine. The purity of the peptide was over 95% by HPLC. The peptide was identified by use of a MALDI-TOF mass spectrometer (HP 1100 Series LC/MSD, Hewlett-Packard, Roseville, USA).

Production of Monoclonal Antibodies. The JN-1-2 mAb used in this study was prepared by using the above synthetic 19-meric polypeptide conjugated with h-IgG.

At first, female BALB/c mice were subcutaneously injected with 100 μg/mouse of the conjugate (1.0 mg/mL in PBS), which was emulsified with an equal volume of Titer Max Gold (Funakoshi Co. Ltd., Tokyo). At second and third injections (20 and 36 days after the first injection, respectively), 100 μg/mouse of the conjugate emulsified with Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, MI, USA) was subcutaneously administered. At the fourth injection, 100 μg/mouse of the conjugate emulsified in Freund's incomplete adjuvant (FIA) (Difco Laboratories) was subcutaneously administered 56 days after the first injection. A final dose of 100 μg/mouse of the peptide-hIgG in 100 μL of PBS was intravenously injected 65 days after the first injection through the tail vein of each respondent mouse three days before the fusion. The immunized spleen cells removed from the mice were fused with myeloma SP/NSI/1-Ag4−1 (NS-1) at a ratio of 5:1 using 50% PEG 1500 (Boehringer Mannheim GmbH, Germany). The fused cells were placed into the wells of 96-well culture plates (Becton Dickinson and Co., Franklin Lakes, NJ, USA) and cultivated in HAT medium. The fused cells were screened to find the antibody-secreting cells by means of modified sandwich ELISA where a peptide (TGLRNGITNKVNSVIEKAA) was coated as the antigen for accurate screening of hybridomas. Hybrids that were found to secrete antibodies specific for the peptide were cloned more than three times by the limiting dilution method. The isotypes of the resulting monoclonal antibodies were determined using a Mouse Monoclonal Antibody Isotyping Kit (IsoStrip, Roche 1493027, Indianapolis, IN, USA, or Amersham Pharmacia Biotech UK Ltd.). Ascites fluid was obtained by intraperitoneal injection of the hybridoma cell lines in pristane-primed female BALB/c mice.

Enzyme-Linked Immunosorbent Assay (ELISA). An amount of 50 mL of antigenic peptide, irrelevant peptides, and other proteins dissolved in PBS solution (4 μg/mL) was fixed on an immuno plate (Nunc, Denmark) at 4 °C overnight. Blocking was performed using 2% gelatin for 1 h at room temperature. After the plate was washed, JN1-2 mAb was immunoreacted, followed by a reaction with antimouse Ig (G+A+M) conjugated with alkaline phosphatase. After the substrate reaction using *p*-nitrophenyl phosphate, the absorption band at 405 nm was measured by use of an ImmunoPlate Reader (Immuno Mini, NJ-2300, Nalge Nunc International K.K., Tokyo, Japan).

Sequencing and Molecular Modeling. Messenger RNA was isolated from the hybridoma-secreting JN1-2 mAbs using a QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech). The cDNAs of the light and heavy chains were synthesized by a first-strand cDNA Synthesis Kit (Life Science Inc., FL, USA). The VH and VL fragments were amplified directly by adding them to a mixture containing PCR components (PCR Thermal Cycler PERSONAL, Takara, Kyoto) and mouse Ig primers specific for IgG (Novagen/Mouse Ig Primer Kit, Darmstadt, Germany). The amplified DNA was visualized on 2.0% agarose gel containing 0.5 μg/mL of ethidium bromide. A band of approximately 450 bp was observed, which corresponds to the size of the variable fragment of the antibody gene with little or no extraneous product. The PCR product was cloned into a pCR4-TOPO Vector system (Invitrogen, Carlsbad, CA). Sequencing was conducted using an Auto Read Sequencing Kit (Amersham Pharmacia Biotech) and an automated DNA sequencer (CEQ8000, Beckman Coulter, Inc. CA). Detailed reaction conditions of PCR were: (1) preheating at 95 °C for 10 min, (2) denaturation at 94 °C for 1 min, (3) annealing temperature at 50 °C for 2 min, and (4) elongation at 50 °C for 2 min. This cycle was repeated 40 times. Finally, elongation was again conducted at 72 °C for 10 min. The primer used was for N-terminal/mouse Ig V_H5′-E, (mixture of ACTAGTCGACATGGGATGGAGCTRTATCATSYTCTT, ACTAGTCGACATGAAGWTGTGGBTRAACTGGRT, and ACTAGTCGACATGGRATGGASCKKIRTCTTTMTCT) for C-terminal/mouse Ig V_H3′-2(CCCAAGCTTCCAGGGRCCARKGGATARACIGRTGG).

Computational analyses of the antibody structures were performed using the deduced VL and VH amino acid sequences with a workstation (Octane 2, Silicon Graphics Inc., PA, USA) running AbM software (Oxford Molecular Ltd., Oxford, UK), which is used for building models of three-dimensional molecules. The resulting PDB data were applied to minimize total energy by using DS-Modeling (Accelrys Software Inc., San Diego, CA, USA). This software uses the CHARMm algorithm for minimizing the energy of a molecule.[19] Protein Adviser Ver. 3.5 (FQS Ltd., Fukuoka, Japan) was employed to visualize, analyze, and draw the structures.

Expression of HA_2 Subunits. CDNA of the HA_2 domain of hemagglutinin (H1-subtype) from the influenza virus (A/Hiroshima/37/2001 (H1N1)) was synthesized by using a polymerase chain reaction (PCR) procedure in which the following primers were employed: forward primer (5′-ACACACATATGGGTTTGTTTGGAGCCAT-3′), which contains an *Nde*I site (underlined), and the reverse (5′-AAAAAACTCGAGGATGCATATTCTACACT-3′), which contains an *Xho* I site (underlined). An aliquot of the PCR mixture was analyzed by agarose gel electrophoresis. To construct a plasmid for the expression of the recombinant HA_2 domain protein, the PCR-amplified DNA fragment was

ligated to the expression vector, pET21a(+) (Novagen, Madison, WI, USA). *Escherichia coli* (Rosseta-gami (DE3)) was transformed and induced the HA_2 domain protein by an addition of a final concentration of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG). The molecular weight of the protein thus obtained was estimated to be 27 kDa by agarose gel electrophoresis.

Purification of the Antibody and Isolation of the Heavy Chain. JN1-2 mAb ($IgG_1(k)$) was purified according to the instruction manual for the Bio-Rad Protein A MAPS-II Kit (Nippon BIO-RAD, Tokyo, Japan). First, 5 mL of ascites fluid containing JN1-2 mAb was mixed with the same volume of a saturated solution of ammonium sulfate. The precipitate was recovered by centrifugation, and then 5 mL of PBS was added to the precipitate. This process was repeated twice, followed by two dialyses against PBS. An aliquot of the PBS solution containing JN1-2 mAb was mixed with the same volume of the binding buffer of MAPS-II. This mixture was then placed on a bed packed with Affi-Gel (protein A) for elution of the bound mAb. The eluted mAb was dialyzed twice against the buffer, 50 mM Tris/0.15 M NaCl (pH 8.0), at 4 °C. The resulting antibody was ultrafiltered three times by use of Centriprep 10 (Amicon, MA, USA). A total of 5 mg of the antibody was dissolved in 2.7 mL of a buffer (pH 8.0) consisting of 50 mM Tris and 0.15 M NaCl and reduced by the addition of 0.3 mL of 2 M 2-mercaptoethanol for 3 h at 15 °C. To this solution, 3 mL of 0.6 M iodoacetamide was added, followed by adjusting the pH to 8.0 by adding 1 M Tris. The solution was then incubated for 15 min at 15 °C. The resulting solution was ultrafiltered to 0.5 mL, after which a half volume of the sample was injected into an HPLC column (Protein-Pak 300SW, 7.8 × 300 mm, Nippon Waters, Tokyo, Japan) at a flow rate of 0.15 mL/min of 6 M guanidine hydrochloride (pH 6.5) as an eluent. Fractions for the heavy and light chains were collected followed by dilution with 6 M guanidine hydrochloride. These fractions were dialyzed against PBS by replacing the buffer seven times for 3−4 days at 4 °C. Detailed procedures are described in the references.[3,9,17,18]

Cleavage Assays. Before the degradation reaction of the antigenic peptide, TGLRNGITNKVNSVIEKAA, by the heavy chain of JN1-2 mAb (JN-2-H) was conducted, most glassware, plasticware, and buffer solutions were sterilized by heating (180 °C, 2 h), autoclaving (121 °C, 20 min), or passing through a 0.2 μm sterilized filter. Manipulations in the experiment were mostly performed in a biological safety cabinet to avoid airborne contamination.

The degradation reaction was conducted in a 15 mM phosphate buffer (pH 6.5) at 25 °C. For monitoring the reaction, 20 μL of the reacting solution was injected into RP- HPLC (Jasco) under isocratic conditions (0.05% trifluoroacetic acid and 12.5% acetonitrile) with a column temperature of 40 °C.

The reaction products were analyzed by using a mass spectrometer (MALDI-TOF-MASS, Bruker/Autoflex, Germany).

In accordance with refs 3 and 9, JN1-2-H, which had been applied to the peptide cleavage assay, kinetic analysis was used for assays against the influenza virus.

In the kinetic analysis, the concentration of JN1-2-H was fixed at 0.05 μM; that of the antigenic peptide varied from 50 to 320 μM at 25 °C in the phosphate buffer (pH 6.5). The change of concentration of the antigenic peptide within 14−40 min (after the addition of the peptide substrate) was regarded as the initial rate of the reaction.

Degradation of Recombinant HA_2 (rHA_2) Labeled with a Biotin by JN1-2-H. The expressed and purified recombinant HA_2 (rHA_2) was submitted for labeling with a biotin molecule, where a reagent of Biotin-$(AC_5)_2$Sulfo-Osu (Dojin, Kumamoto, Japan) was used in accordance with the protocol. The band of the biotinylated rHA_2 was detected at 28 kDa in the SDS-PAGE analysis after the reaction of POD-labeled streptavidin with Biotin-rHA_2.

The degradation reaction was performed in a phosphate buffer (pH 6.5) containing 0.1% Triton X under concentrations of 71 nM of Biotin-rHA_2 (Biotin-rHA_2) and 0.4 μM of JN1-2-H and a reaction temperature of 25 °C. The time course of degradation of rHA_2 was monitored by Western blot analysis after SDS-PAGE (12% gel) of the reaction products, which were taken from the reaction vessel at each incubation time and stored at −30 °C until the SDS-PAGE experiment. After SDS-PAGE (12% gel) was carried out without staining, the proteins were transferred from the gel onto an Immobilon-P PVDF membrane (30 V, 180 mA, 90 min). The PVDF membrane (Millipore, Cat. No. IPVH00010, Lot No. K5BN2081A) was blocked with TBS containing 3% skim milk and 0.05% Tween 20. After being washed with TBS containing 0.05% Tween 20 (TBS-T), the mixture was incubated with streptavidin conjugated with peroxidase (1/300 dilution) for 1 h at room temperature. After washing with TBS-T, color development was performed for 6 min by using BCIP/NBT (Kirkegaard & Perry Laboratories, Maryland, USA).

Assay for Lysed Influenza Virus. The influenza A virus (A/Hiroshima/37/2001(H1N1)) obtained by egg culture (see Methods) was prepared and treated with detergent (1% Triton X-100) to inactivate the virus (lysed virus) for the safety of the experimenters. A solution of JN1-2-H (0.7 mL/0.4 μM) was mixed with 0.7 mL of lysed virus (H1N1: ca. 5×10^{13} particles/mL) in 15 mM phosphate buffer (pH 6.5) and was incubated at 25 °C. Degradation was monitored by Western blot analysis using POD-labeled JN1-1 mAb (1000 dilution) after SDS-PAGE (12% gel) of the reaction sample had been performed. Color development after the immunoreaction of the POD-labeled JN1-1 mAb was made using TrueBlue (BNCP/NBT phosphatase substrate) for 5 min.

Immunoblot Analysis. After SDS-PAGE was performed without staining, the proteins were transferred from the gel onto an Immobilon-P PVDF membrane. The PVDF membrane was blocked with TBS containing 3% skim milk and 0.05% Tween 20. After being washed with TBS containing 0.05% Tween 20 (TBS-T), the membrane was incubated with JN1-1 mAb conjugated with peroxidase for 1 h at room temperature.[13] After the washing with TBS-T, color development was performed by using BCIP/NBT (Kirkegaard & Perry Laboratories, MD, USA).

In Vitro Assay. *Virus.* The influenza viruses used were A/Hiroshima/37/2001 (H1N1) and B/Hiroshima/22/2001. These viruses were grown in allantoic cavities of 11-day-old embryonated chicken eggs, harvested, and stored as infectious allantoic fluid at −80 °C until further use.

Cells. MDCK cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum.

Neutralization Tests. Neutralization tests were performed as follows.[20]

The monoclonal antibody or antibody heavy chain diluted with phosphate-buffered saline (PBS) was mixed with an equal volume (250 μL) of influenza virus diluted with Eagle's MEM medium adjusted to give a final control count of about 500 PFU or 5000 PFU per 0.2 mL. After incubation for 48 h at 20 °C, an infectious virus titer of the mixture was calculated by a plaque assay. The antibody- or heavy chain-virus mixture was serially diluted in ten steps: 0.2 mL of each mixture was inoculated into the MDCK cell monolayer, which had been seeded on a 6-well tissue culture tray (Falcon 3046; Becton Dickinson Labware, Oxnard, CA, USA). After adsorption for 60 min at 37 °C, the inocula in each well were removed and washed with PBS. The MDCK cells were covered with the first overlay MEM medium containing 1.0% agarose ME (Iwai Chemical Industries, Tokyo, Japan) and 20 mg of trypsin (Merck) per milliliter, and the trays were incubated four days in a humidified 5% CO_2 incubator at 34 °C. After incubation, the cells were covered with the second overlay MEM medium (0.005% neutral red in the first overlay medium). The plaques were counted on the following day.

RESULTS

Design of Antigenic Peptide for Immunization and the Production of mAbs. Okuno et al.[14] have already reported that

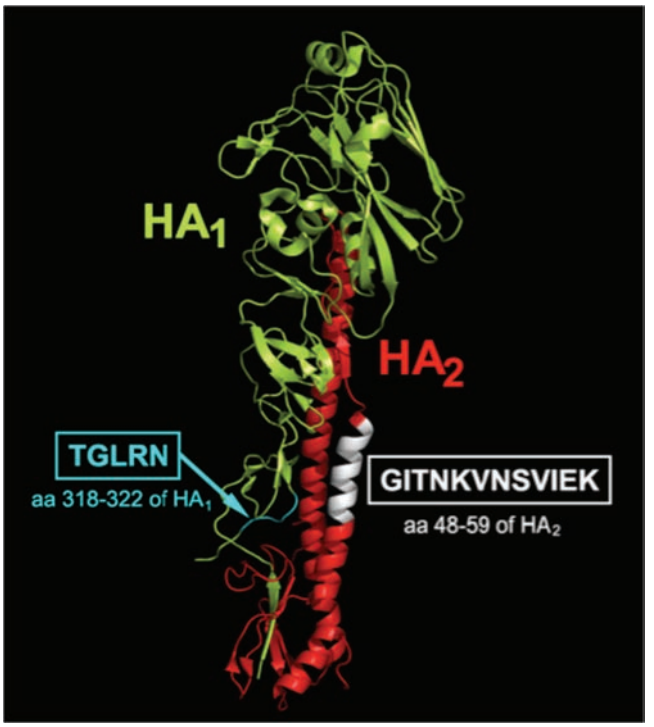

Figure 1. Location of conserved sequences in hemagglutinin. The sequence TGLRN (aa 318−322) is present in the HA_1 domain and that of GITNKVNSVIEK (aa 48−59) in the HA_2 domain of the hemagglutinin molecules of H1N1 influenza virus type A. HA_1 domain: green. HA_2 domain: red. TGLRN: blue. GITNKVNSVIEK: white. In this study, the above two sequences were combined and synthesized as one antigenic peptide, TGLRNGITNKVNSVIEKAA, in which two alanine residues were adducted at the C-terminal.

the monoclonal antibody (mAb), C179, neutralizes the infection of H1N1 of influenza virus type A. The epitope was determined as TGLRN present in the HA_1 domain and GITNKVNSVIEK in the HA_2 domain. Their locations are shown in Figure 1. The sequence TGLRN (aa 318−322 in the HA_1 domain) is conserved in many subtypes such as H1, H2, H6, and H13. The sequence GITNKVNSVIEK (aa 48−59 in the HA_2 domain) is conserved in only the H1 and H2 subtypes but not in the others. The C179 mAb recognizes and binds with both sequences at the same time. We combined the above two peptides into a single peptide, TGLRNGITNKVNSVIEK. The peptide does not have a helix conformation, which was determined using a computer. Thus, we adducted two alanine residues at the C-terminal of the combined peptide (TGLRNGITNKVNSVIEKAA), which was used as the antigenic peptide in this study. The peptide was conjugated with h-IgG via a Cys residue introduced at the C-terminal of the antigenic peptide and injected into BALB/c mice.

After the injection, two monoclonal antibodies (mAbs: JN1-1 and JN1-2; $IgG_1(k)$) were established using the conventional hybridoma generation technique.[15,16] As shown in Figure 2a, the mAbs that were obtained reacted specifically with the antigenic peptide but not with some irrelevant proteins such as human γ-globulin (h-IgG), BSA, HSA, KLH, and human hemoglobin (h-Hb) and some irrelevant peptides such as TPRGPDRPEGIEEEGGERDRD (TP41-1: a part of gp41 of HIV), RSSHFPYSQYQFWKNFQTLKGC (ECL2A: a part of CCR-5), CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC (V3 loop: a part of the V3 loop of HIV), and KLLRGTKALTFVIPLTEEAE (RT-1: a part of the reverse transcriptase of HIV). The mAbs did react with influenza virus type A H1N1 (A/Hiroshima/37/2001) but only slightly with H2N2 (A/Singapore/1/57) and H3N2 (A/Hiroshima/71/2001). The JN1-2 mAb was confirmed to react with the recombinant HA_2 domain based on Western blot analysis (data not shown).

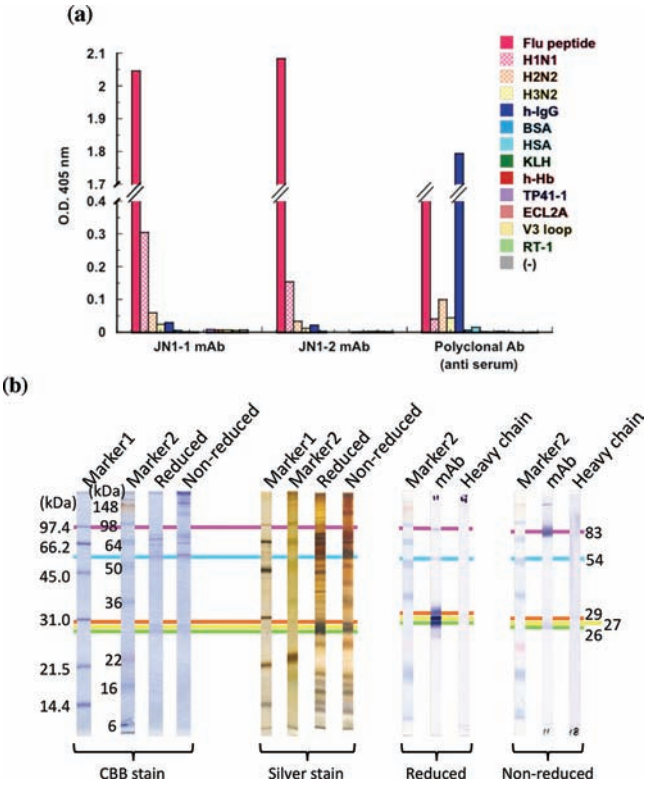

Figure 2. Cross reactivities of mAbs with some viruses, proteins, and peptides by ELISA. (a) Cross reactivities. Influenza viruses type A H1N1 (A/Hiroshima/37/2001), H2N2 (A/Singapore/1/57), and H3N2 (A/Hiroshima/71/2001) were tested. Some irrelevant proteins such as human γ-globulin (h-IgG), BSA, HSA, KLH, and human hemoglobin (h-Hb) were examined. As irrelevant peptides, TPRGPDRPEGIEEEGGERDRD (TP41-1: a part of gp41 of HIV), RSSHFPYSQYQFWKNFQTLKGC (ECL2A: a part of CCR-5), CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC (V3 loop: a part of V3 loop of HIV), and KLLRGTKALTFVIPLTEEAE (RT-1: a part of reverse transcriptase of HIV) were also examined. JN1-1 and JN1-2 mAbs strongly reacted with the antigenic peptide and H1N1 virus. It seems that the mAbs reacted very slightly with H2N2 (and H3N2). (b) SDS-PAGE and Western blot for the H1N1 virus. First, SDS-PAGE using lysed H1N1 virus was performed, followed by CBB and silver staining. Second, another SDS-PAGE using the same lysed virus was performed to prepare strip membranes (3 mm × 60 mm/each) for Western blot analysis, in which JN1-2 mAb (mAb; 0.25 μg/mL) or JN1-2-H (heavy chain; 50 μg/mL) was used as the first antibody. As the second antibody, ALP-conjugated goat $F(ab')_2$ to mouse IgG/A/M (Cappel; 1/1000 dilution) was used. For color development, a BCIP/NBT phosphatase substrate (KPL) was employed. SDS-PAGE: Stacking gel 3%, running gel (12%). Marker1: Low-range marker (BIO-RAD). Marker2: SeeBlue prestained standard marker (Invitrogen).

In contrast, the polyclonal antibody (antiserum) obtained at the cell fusion of immunized mice showed a strong reactivity to the antigenic peptide and human IgG as expected.

The heavy (JN1-2-H) chain was separated from the parent monoclonal antibody (JN1-2 mAb) and purified in accordance with refs 3, 9, and 18. The immunoreactivities of JN1-2-H and

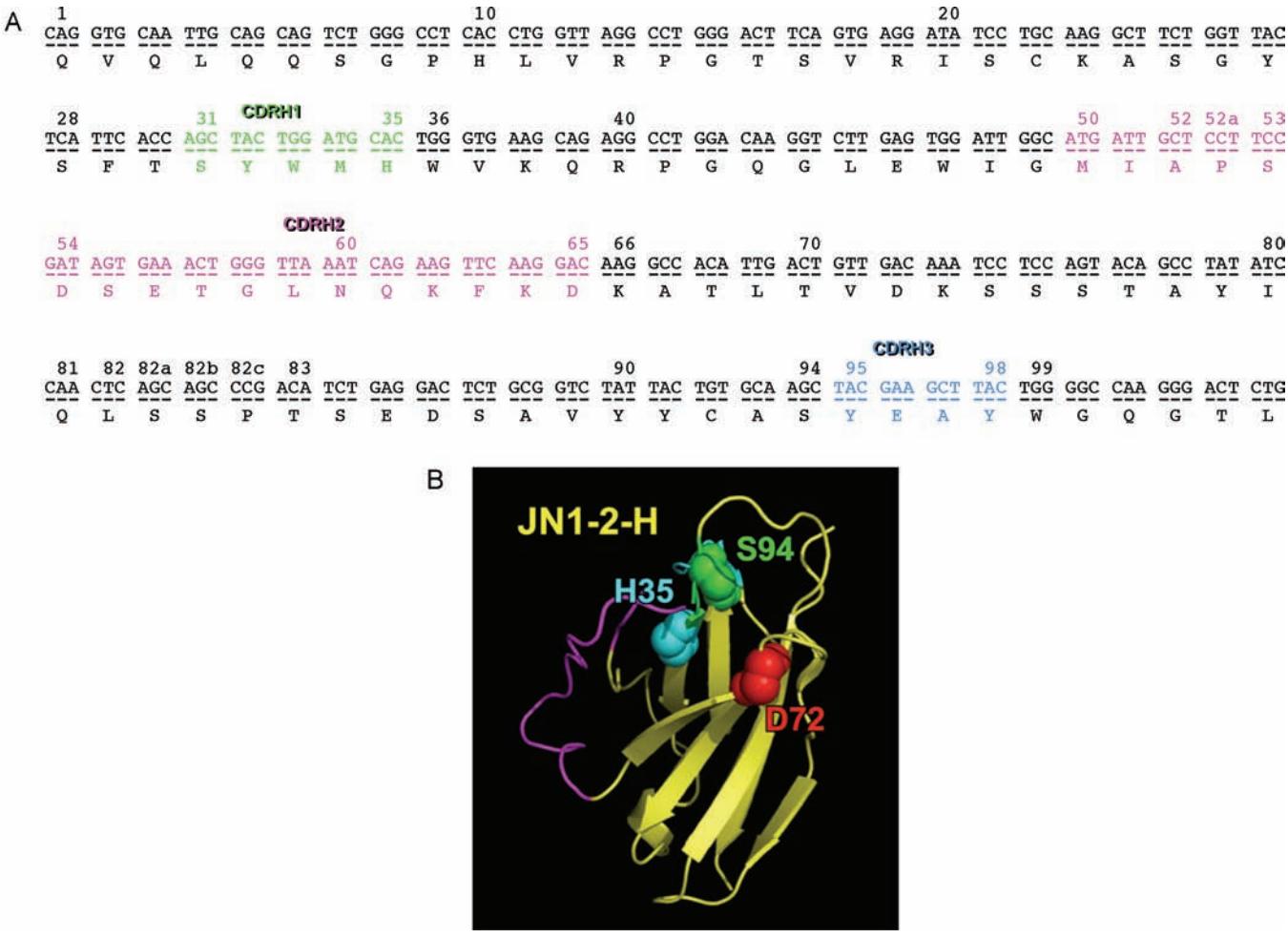

Figure 3. Sequence and structure of JN1-2-H. The sequence of cDNA of JN1-2-H was determined, and the germ line gene of JN1-2-H was identified as J558.46 (DDBJ/EMBL/GENBANK: accession No. AB570293) (Figure 3a). It is characteristic that the CDR-3 region consisted of only four amino acid residues. The structure of the variable region of JN1-2-H was created by molecular modeling using the deduced aa sequence. Three amino acid residues (His35, Asp54, and Ser94) are located close to each other and might function as a catalytic triad in a manner similar to other cases.[14,15] It seems that the distance between His and Ser is of importance. The Cα distance between His and Ser is 8.42 Å in a catalytic triad of trypsin. The distance in JN1-2-H might be preferable for constructing such a triad.

JN1-2 mAbs for the H1N1 virus were investigated using Western blot analysis along with SDS-PAGE, as shown in Figure 2b. The inactivated virus (H1N1) was submitted to SDS-PAGE and then stained with CBB or silver. In CBB staining, two bands at 26 (green line) and 27 kDa (yellow line) corresponding to the HA_2 domain were obvious under reduced conditions and weakly visible under nonreduced conditions. A band at 54 kDa (blue line) was considered a dimer of HA_2. In silver staining, the bands at 26, 27, 54, and 83 kDa were obvious. The band at 83 kDa might be a trimer of HA_2 or HA_0 (or their mixture), although it is not clear at present. In addition to these bands, many other bands were observed. Western blot analysis using JN1-2 mAb or the heavy chain (JN1-2-H) indicated some clear bands. Under reduced conditions, JN1-2 mAb showed strong bands at 26 (green line) and 27 kDa (yellow line) and a weak band at around 29 kDa (orange line), which correspond to the HA_2 domain of hemagglutinin. The mAb showed a strong band at 83 kDa. Under nonreduced conditions, a faint band at 29 kDa in the case of JN1-2-H was observed. Under reduced conditions, it was observed as a weak band at 27 kDa. Although the details are not yet clear, it seems that the conformation of the hemagglutinin molecule may take several forms under reduced conditions.

On the basis of ELISA (data not shown), the affinity constant of the mAb to the antigenic peptide and the recombinant HA_2 domain (which was expressed in *E. coli* and recovered; see Methods) was 1.8×10^8/M and 6×10^7/M, respectively. Although similar experiments were performed for JN1-2-H, we could not obtain a precise value. Roughly estimated, the affinity constant against the antigenic peptide was 2 orders of magnitude less than that of the mAb.

Nucleotide Sequencing and Molecular Modeling. Sequencing of the cDNAs of the variable region of the light chain and heavy chain (JN1-2-H) of JN1-2 mAb was conducted, and the amino acid sequences were deduced (Figure 3a). The germ line gene of JN1-2-H was identified as J558.46 (DDBJ/EMBL/GENBANK: accession No. AB570293). It is characteristic that the CDR-3 region consists of only four amino acid residues. The three-dimensional structure of the variable region of JN1-2-H was created by molecular modeling according to the method described in ref 21. The Cα distance between His35 and Asp72 is 14.56 Å, and that between His35 and Ser94 is 6.78 Å. These

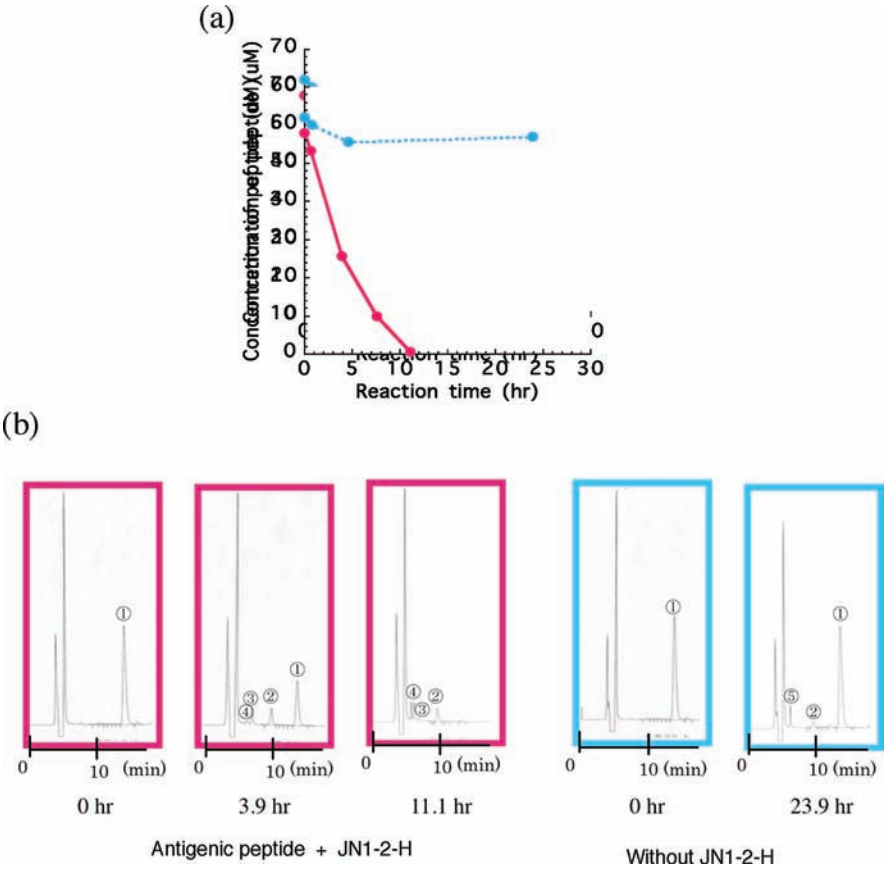

Figure 4. Time course of the catalytic cleavage of an antigenic peptide by JN1-2-H. (a) Peptide (TGLRNGITNKVNSVIEKAA): 60 μM. JN1-2-H: 0.4 μM. The reaction was conducted at 25 °C in a phosphate buffer (pH 6.5). (Pink -●-): Degradation curve for the antigenic peptide by JN1-2-H. (Blue -●-): Time course of the antigenic peptide without JN1-2-H, as a control. The antigenic peptide soon degraded after being mixed with JN1-2-H, while the change hardly occurred without the presence of JN1-2-H. (b) Time course of degradation of the antigenic peptide by RP-HPLC analysis. The antigenic peptide (peak 1) at a retention time of 14 min was cleaved into several small fragments (peaks 2, 3, and 4). Finally, the antigenic peptide completely disappeared. In the control experiment without JN1-2-H, the antigenic peptide hardly cleaved.

distances are very similar to those of the catalytic heavy (or light) chains of HpU-2-H,[18] VIPase,[1] i41SL2-1-L,[22] UA15-L,[9] etc. It seems that the distance between His and Ser is of importance. The Cα distance between His and Ser is 8.42 Å in a catalytic triad of trypsin. The distance in JN1-2-H might be preferable for constructing such a triad. Thus, three aa residues (His35, Asp72, and Ser94 (or Ser31)) are located close to each other in JN1-2-H and are assumed to form a catalytic triad, as shown in Figure 3b.

Cleavage Assay for Peptides. A peptidase activity of JN1-2-H was examined by using the antigenic peptide, TGLRNGITNKVNSVIEKAA, at 25 °C according to the protocol described in the Methods.

RP-HPLC was used to monitor the time course of the cleavage of the antigenic peptide. As shown in Figure 4a, degradation of the antigenic peptide began soon after the peptide and JN1-2-H were mixed together. The antigenic peptide was rapidly cleaved, and at 11.1 h, it was completely decomposed. In contrast, the change hardly occurred without JN1-2-H. Figure 4b shows chromatograms of the RP-HPLC. At a reaction time of 3.9 h, the antigenic peptide at a retention time of 15 min had decreased and cleaved into several small fragments observed at retention times of 10.0, 6.9, and 6.0 min (peaks 2, 3, and 4, TGLRNGITNKVNSVIEKAA, respectively). At 11.1 h, the antigenic peptide had completely disappeared from the reaction system along with a decrease of peaks 2 and 3 and an increase of peak 4. A new peak at a retention time of 5.5 min (peak 5) was observed. Two small peaks of 2 and 5 after 23.9 h of incubation without JN1-2-H were not generated from the fragmentation by JN1-2-H.

We performed the same cleavage assay using an unrelated heavy chain, which was prepared from the parent monoclonal antibody (InfA-6)[23] raised against the other site (GMVDGWYG; aa 17−24) of the HA_2 domain under the same experimental conditions. No cleavage was observed by the heavy chain for the antigenic peptide (TGLRNGITNKVNSVIEKAA) (data not shown).

The light chain (JN1-2-L) possessed a weak cleavage function compared with the heavy chain. Although JN1-2 mAb was also subjected to similar experiments, they failed to cleave the antigenic peptide. By association with the heavy and light chains, the affinity strength must be largely enhanced, but the catalytic function might be hindered. Perhaps the catalytic site is buried between the faces of the heavy and light chains.

The reacted solution sample of antigenic peptide was analyzed by a mass spectrometer. The cleaved bonds of the peptide were determined to be K10−V11, N12−S13, E16−K17, and K17−A18, as shown in Figure 5.

The kinetic data were obtained by varying the concentration of the antigenic peptide while keeping that of JN1-2-H constant at 0.05 μM. The Hanes−Woolf plots (Figure 6) revealed that the reaction by JN1-2-H fit the Michaelis−Menten equation. Thus,

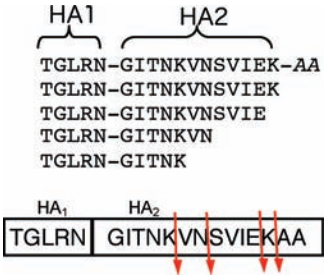

Figure 5. Cleaved bonds of the antigenic peptide by JN1-2-H. The solution after the degradation of the antigenic peptide was analyzed by a mass spectrometer. The cleaved peptide bonds were K10−V11, N12−S13, E16−K17, and K17−A18.

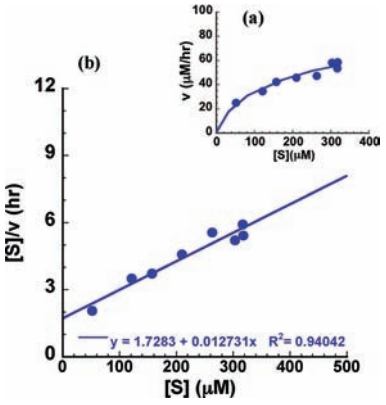

Figure 6. Kinetic analysis. The concentration of JN1-2-H was fixed at 0.05 μM, and that of the antigenic peptide was varied from 50 to 320 μM at 25 °C in phosphate buffer (pH 6.5). (a) Plot of concentration of the antigenic peptide [S_0] vs the initial rate [V]. (b) Hanes−Woolf plot by JN1-2-H. [S]: concentration of the antigenic peptide. [V]: initial rate of the cleavage reaction. This plot demonstrates that the cleavage reaction by JN1-2-H fits the Michaelis−Menten kinetics equation, indicating that the reactions are enzymatic.

the degradation of the antigenic peptide by JN1-2-H should be enzymatic. The dissociation constant (Km) was determined to be 1.36×10^{-4} M. The catalytic reaction constant (kcat) was 26.2 min^{-1}. The catalytic efficiency (kcat/Km) was 1.93×10^5/M^{-1} min^{-1}. The kcat value was very high in comparison with the values of other catalytic antibodies reported so far.[1,3,17]

Degradation of Recombinant HA_2 (rHA_2) Labeled with Biotin by JN1-2-H. As shown in Figure 7, the time course of the cleavage of the Biotin-rHA_2 was monitored at 0, 4, 8, 12, 21, 26, 36, and 48 h of incubation. In this case, SDS-PAGE was performed under the nonreduced condition. The control was also monitored without JN1-2-H (see the results of "only rHA_2").

At 0 h of the incubation of the mixture of Biotin-rHA_2 and JN1-2-H, a clear and strong band appeared at around 28 kDa corresponding to monomeric rHA_2.The band at 28 kDa became fainter with further incubation until 48 h. (It is unclear whether the band became slightly stronger at 26 h of incubation.) On the other hand, the band at 28 kDa in the control experiment hardly changed during 0−48 h of incubation. In the incubation of the mixture of Biotin-rHA_2 and JN1-2-H, a faint band at around 15 kDa was visible after 4 h of incubation, and the band strength reached a plateau at 20−30 h of incubation. The band was considered a fragment generated from rHA_2.

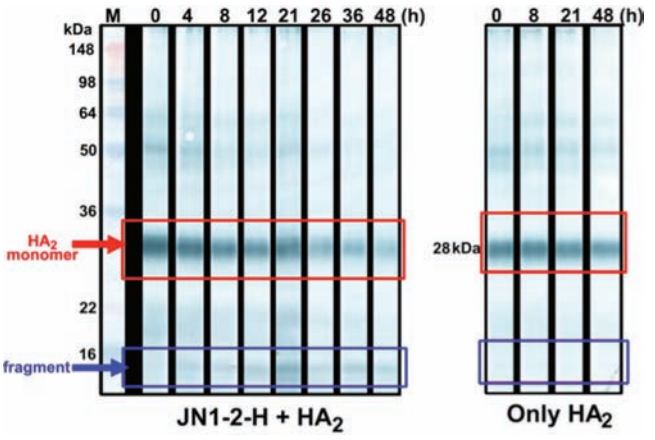

Figure 7. Degradation of recombinant HA_2 (rHA_2) labeled with a biotin by JN1-2-H. A recombinant HA_2 was labeled with a biotin molecule. The biotin-rHA_2. Biotin-rHA_2: 2 μg/mL (71 nM). JN1-2-H: 20 μg/mL (0.4 μM). The reaction was performed in a phosphate buffer (pH 6.5) containing 0.1% Triton X-100 under the reaction temperature of 25 °C. At 0 h of incubation of the mixture of Biotin-rHA_2 and JN1-2-H, a clear, strong band appeared at around 28 kDa, corresponding to monomeric rHA_2. The band at 28 kDa grew fainter with further incubation until 48 h. A faint band at around 15 kDa was visible after 4 h of incubation; the band strength reached a plateau at 20−30 h of incubation. The band is considered a fragment generated from rHA_2. In contrast, the band at 28 kDa in the control experiment hardly changed during 0−48 h of incubation.

No BSA and HSA Cleavage by JN1-2-H. To examine substrate specificity, JN1-2-H (0.2 μM) was incubated with BSA and HSA (0.3 μM) under conditions identical to those employed in the above experiments. No cleavage of BSA and HSA was detected after 24 h of incubation (data not shown). In addition to the experiments, we conducted the degradation experiment in which 50 μM concentration of BSA was mixed with 0.2 μM JN1-2-H. In this case, we could not observe any fragmented band at all.

Assay of Lysed Influenza Virus. Decomposition of the HA_2 domain was monitored by Western blot analysis (12% gel was used in SDS-PAGE) using POD-labeled JN1-1 mAb (not JN1-2 mAb). The results are shown in Figure 8. A band corresponding to the HA_2 domain appeared at around 28 and 29 kDa. After 5 h of incubation of a mixture of the virus (H1N1) and JN1-2-H, both bands had faded considerably. After 24 h of incubation, the bands seemed to have almost disappeared. In the control experiment, the bands were clearly visible for the first 24 h of incubation.

In Vitro Assays. The influenza viruses of A/Hiroshima/37/2001(H1N1) and B/Hiroshima/22/2001 were used in this study. Prior to advancing to this experiment, some incubation conditions (temperature: 4°, 10°, 20°, 27°, and 37 °C; time: 0, 1, 3, 8, 24, 48, 72, 168, and 240 h) were investigated because the influenza virus loses its infectivity at high temperatures. At 37 °C incubation, the virus rapidly lost infectivity. Conversely, few changes were observed at 4 and 10 °C for the first 240 h of incubation. Considering the loss of infectivity of the influenza virus and the catalytic activity of JN1-2-H (as the catalytic function of JN1-2-H is reduced at low temperatures), the most appropriate incubation temperature was determined to be 20 °C and the incubation time 48 h.

Table 1 shows the results of evaluating the neutralization factor of antibodies against influenza viruses, where the in vitro

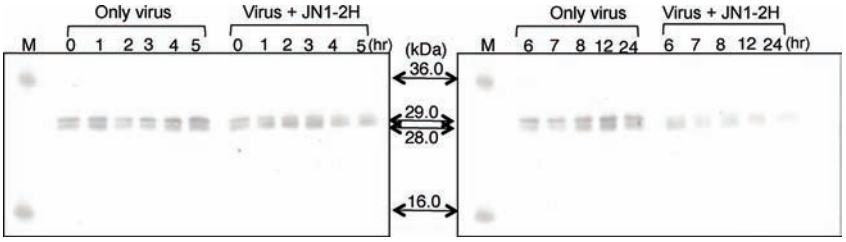

Figure 8. Assay for lysed influenza virus. SDS-PAGE: 12% gel under nonreducing conditions. Influenza virus (lysed with 1% Triton X-100): ca. 5×10^{13} particles/mL. JN1-2-H: 0.2 μM. Reaction volume: 1.4 mL. Detection: POD-labeled JN1-1 mAb. Reaction of JN1-2-H with the lysed virus (H1N1) was conducted in 15 mM phosphate buffer (pH 6.5) at 25 °C. Degradation was monitored by Western blot analysis using POD-labeled JN1-1 mAb after SDS-PAGE (12% gel) had been performed. A band corresponding to the HA_2 domain appeared at around 28 and 29 kDa and became faint with incubation time. After 5 h of incubation, both bands at around 28−29 kDa had faded considerably; after 12 h of incubation, the bands seemed to almost disappear. In the control experiment without JN1-2-H, the bands were clearly visible even after 24 h of incubation.

Table 1. Results of Neutralization Tests by JN1-2 mAb and JN1-2-H

catalytic antibody or mAb	virus strain	neutralization factor[a] (dilution of catalytic antibody or mAb)	concentration (μM)
JN1-2-H	**A**/Hiroshima/37/2001(H1N1)	4	0.35
	B/Hiroshima/22/2001	<2	>0.70
JN1-2-mAb	**A**/Hiroshima/37/200(H1N1)	<2	>0.65 (ligand)

[a] 50% inhibition for the infection of the influenza virus.

Table 2. Results of Reduction of Number of the H1N1 Virus[a]

influenza virus	antibodies	concentration of antibody (μg/mL)	numbers of virus (PFU/0.2 mL) initially prepared virus (PFU/0.2 mL)	incubation at 20 °C for 48 h: without antibodies	incubation at 20 °C for 48 h: with antibodies	degree of suppression of infectivity (%)
type A (H1N1)	no antibody (control)	0	5×10^3	1.3×10^3 ($\pm 2.8 \times 10^2$) [b]	/	–
		0	5×10^2	1.7×10^2 ($\pm 3.5 \times 10$) [b]	/	–
	JN1-2-H	25	5×10^3	1.3×10^3 ($\pm 2.8 \times 10^2$) [b]	7.5×10^2 ($\pm 3.5 \times 10^2$)	42.3
		25	5×10^2	1.7×10^2 ($\pm 3.5 \times 10$) [b]	5.5×10^1 ($\pm 7.1 \times 10^0$)	67.6
		2.5	5×10^3	1.3×10^3 ($\pm 2.8 \times 10^2$) [b]	1.3×10^3 ($\pm 2.1 \times 10^2$)	0
		2.5	5×10^2	1.7×10^2 ($\pm 3.5 \times 10$) [b]	1.2×10^2 ($\pm 2.8 \times 10^1$)	29.4

[a] Each assay was performed with $n = 2$. [b] These values were used as a standard to calculate the degree of infectivity.

assays were repeated three times. Considering the minimum concentration of antibody or catalytic antibody (Table 1), JN1-2-H could suppress the infection of the H1N1 virus of A type at the minimum concentration of 0.35 μM but not against type B virus. The parent antibody, JN1-2 mAb, did not show any neutralizing effect against the type A virus.

Next, further experiments were performed to get more precise data, as shown in Table 2.

In the experiment, cases without antibodies were performed with the number of viruses equaling 5×10^3 or 5×10^2 PFU/0.2 mL, as the control. After 48 h of incubation, the number of viruses had been reduced by a factor of 4 compared to the initial value, as shown in the table.

When 25 μg/mL of JN1-2-H was mixed with the virus, the suppression of the infection was clearly observed, as presented in Table 2. For the case of 5×10^3 (PFU/0.2 mL) initially, JN1-2-H reduced the number of viruses from 1.3×10^3 to 7.5×10^2 PFU after 48 h of incubation (a reduction of almost half). In the case of 5×10^2 PFU initially, the number was reduced from 1.7×10^2 to 5.5×10^1 PFU (a reduction of almost 2/3). However, at a JN1-2-H concentration of 2.5 μg/mL, a slight effect was observed when the initial number of viruses equaled 5×10^2 PFU/0.2 mL, but no effect was observed when it was 5×10^3 PFU/0.2 mL.

DISCUSSION

Monoclonal antibody JN1-2 was produced by immunization of an antigenic peptide, TGLRNGITNKVNSVIEKAA, in which TGLRN and GITNKVNSVIEK are the conserved amino acid sequences located in the HA_1 and HA_2 domains, respectively, of the hemagglutinin molecule of the H1 and H2 strains of the influenza virus A type. In this study, the above two conserved peptide sequences were combined and synthesized as one peptide at whose C-terminal two alanine residues were introduced to maintain the helical conformation.

JN1-2 mAb specifically reacted with the immunized peptide (antigenic peptide), the recombinant HA_2, and the native HA_2 domain of the H1N1 subtype of influenza virus A type. The amino acid sequence of the variable region (VH and VL) of

JN1-2 mAb was deduced from the cDNA sequence of the mAb. The conformation of JN1-2-H was created using molecular modeling, as shown in Figure 3b.

On the basis of the homology between antibody light chains and serine proteases, Erhan et al. suggested that the light chain of the antibody could function as a peptidase/protease[24] by itself. It has been reported that the active site composed of a catalytic dyad or triad of the catalytic antibody can function to hydrolyze antigens. Kolesnikov et al. reported that the antibody possessing the catalytic dyad (His35 and Ser99) in the heavy chain is the active site to hydrolyze the acetylthiocholine molecule.[25] In addition, Paul et al. revealed that the catalytic triad composed of Asp1, Ser27a, and His93 in the light chain could catalytically hydrolyze the antigen, vasoactive intestinal peptide (VIP).[26] In other catalytic antibody light chains such as i41SL1-2[22] and UA15-L,[9] the identical amino acids are seen. Regarding JN1-2 mAb, it seems that two or three candidates capable of forming a catalytic triad (composed of His35, Asp72 (or Asp54), and Ser94 (or Ser 31)) are encoded in the heavy chain, as shown in Figure 3a and 3b. (DDBJ/EMBL/GENBANK: accession No. AB570293). The position of His35 might be important because the catalytic antibody reported by Kolesnikov et al.[25] also possessed the identical histidine residue. We tried the mutation of these amino acids in JN1-2-H, but we could not obtain any mutated JN1-2-H protein because of the high toxicity of the gene of JN1-2-H against several host cells although several plasmids were used.

On the basis of the cleavage assay, JN1-2-H catalytically decomposed the antigenic peptide. The cleaved bonds of the antigenic peptide by JN1-2-H were at K10−V11, N12−S13, E16−K17, and K17−A18. These cleaved peptide bonds were present in a portion of GITNKVNSVIEK but not in TGLRN. The sequence TGLRN is the conserved sequence of the HA_1 domain. In contrast, GITNKVNSVIEK is conserved in the HA_2 domain. JN1-2 mAb reacted with the HA_2 but not with the HA_1 domain based on Western blot analysis, suggesting that the specificities of JN1-2 mAb and the heavy chain, JN1-2-H, are similar and that they recognize the HA_2 but not the HA_1 domain. In the breakdown of HA_2 (28 kDa) by JN1-2-H, a fragment of 15 kDa was observed. The fragment might have been generated by a successive reaction of the 21 kDa fragment, which is supposed to be broken down first considering the location of the antigenic peptide in the HA_2 domain and which is faintly observed in Figure 7.

There have been reports of natural antibodies capable of cleaving several peptide bonds. Kaveri et al. reported that several peptide bonds were cleaved in their natural catalytic antibody for factor VIII.[5] Paul et al. also reported multiple cleavage sites in HIVgp120 by a catalytic IgM monoclonal antibody.[4] JN1-2-H was also capable of peptide bond cleavage at multiple sites.

From a kinetic study using antigenic peptide, the kcat value of the catalytic JN1-2-H showed a high initial rate (26.2 min^{-1}) compared with those obtained thus far. From the degradation data of Figure 8, 3.5×10^{13} particles (in 1.4 mL of reaction solution) of the influenza virus were degraded within about 24 h by 0.2 μM JN1-2-H. Specifically, one HA_2 molecule was degraded by one JN1-2-H in about 7 min if all hemagglutinin molecules of the virus were decomposed (assuming that about 1000 molecules of HA_2 are present on one virus, there are 3.5×10^{16} molecules of HA-2). The degradation rate of HA_2 of the influenza virus by JN1-2-H was much slower than that of the antigenic peptide. However, in the in vitro assay, JN1-2-H exhibited suppression of the infectivity of influenza virus H1N1-(A/Hiroshima/37/2001) during 48 h at 20 °C. Considering that the parent JN1-2 mAb did not display any effect, the suppression phenomena must be attributed to the catalytic feature of JN1-2-H. Note that JN1-2-H did not show any effect on the infectivity of influenza virus B type. These results showed that JN1-2-H could catalytically and specifically degrade the target protein, the HA_2 domain of H1N1, resulting in the loss of influenza virus infectivity.

In the case of H5N1 (high-risk type), the sequence of its HA_2 domain is GVTNKVNSIIDK. (The H6 subtype also has the identical sequence.) As three amino acids (shown underlined) are different from those of H1N1, JN1-2-H has the possibility of degrading the hemagglutinin of the H5N1 subtype. A study of this possibility will be conducted in the near future.

CONCLUSION

We have succeeded in the preparation of a catalytic antibody heavy chain, JN1-2-H, capable of specifically digesting an antigenic peptide, TGLRNGITNKVNSVIEK, of the HA_2 domain of the hemagglutinin molecule of influenza A virus. In addition, JN1-2-H could suppress the infectivity of the H1N1 subtype of influenza A virus with its catalytic function.

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ABBREVIATIONS:

aa, amino acid; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; HSA, human serum albumin; h-IgG, human IgG; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; MDCK, Madin−Darby canine kidney; pAb, polyclonal antibody; PB, phosphate buffer; PFU, plaque-forming unit; POD, horse radish peroxidase; PVDF, polyvinylidene difluoride; RP−HPLC, reversed phase−high performance liquid chromatography; VH, variable region of heavy chain; VL, variable region of light chain

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