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Formatted single-domain antibodies can protect mice against infection with influenza virus (H5N2)

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ABSTRACT

This work continues a series of recently published studies that employ recombinant single-domain antibody (sdAb, or nanobody®) generation technologies to battle viruses by a passive immunization approach. As a proof of principle, we describe a modified technique to efficiently generate protective molecules against a particular strain of influenza virus within a reasonably short period of time. This approach starts with the immunization of a camel (*Camelus bactrianus*) with the specified antigen-enriched material presented in as natural a form as possible. An avian influenza virus A/Mallard/Pennsylvania/10218/84 (H5N2) adapted for mice was used as a model source of antigens for both the immunization and phage display-based selection procedures. To significantly increase activities of initially selected monovalent single-domain antibodies, we propose a new type of sdAb formatting that involves the addition of a special type of coiled-coil sequence, the isoleucine zipper domain (ILZ). Presumably, the ILZ-containing peptides adopt trimeric parallel conformations. After the formatting, the biological activities (virus neutralization) of the initially selected anti-influenza virus (H5N2) sdAbs were significantly increased. Intraperitoneal or intranasal administration of the formatted sdAb at 2 h before or 24 h after viral challenge specifically protects mice from lethal infection with influenza virus. We hope that the described approach combined with the selection focused on particular conservative epitopes will lead to the generation of sdAb-based molecules protective against a broad spectrum of influenza virus subtypes.

1. Introduction

Influenza A and B viruses pose a current pandemic threat to the human population around the world. Sporadic transmission of these viruses from birds (or potentially from swine or other animals) could occur and lead to unpredictable pandemic outbreaks. The antigenic shift of the viral surface protein hemagglutinin makes it difficult to establish a standard set of protective antiviral vaccines or a protective antibody-based approach for passive immunization. There is an urgent need for a reliable universal method of efficiently generating protective molecules against a particular newly emergent strain of influenza virus within a reasonably short period of time (within a few months). The present work describes the proof of principle of one such method, which is based on the generation of virus-neutralizing, recombinant single-domain antibodies. This work continues a series of similar, recently published studies employing recombinant single-chain or single-domain antibody generation technologies to battle viruses (Hultberg et al., 2011; Ibañez et al., 2011; Vanlandschoot et al., 2011). The term "single-domain antibody" (VHH, "nanobody®"(Ablynx), nanoantibody) was given to single-domain variable fragments of a special type of antibody (heavy chain-only antibody, hcAb, which lack light chains) that naturally exist along

Abbreviations: Ab, antibody; sdAb, single-domain antibody; fsdAb, formatted single-domain antibody; HA, influenza virus hemagglutinin; HA1, membrane-distal, globular domain of hemagglutinin; aHAsdAb, anti-hemagglutinin sdAb; aHAfsdAb, anti-hemagglutinin formatted sdAb; hcAbs, *Camelidae*-specific heavy chain-only antibodies (they lack the classical light-chain and are composed of a homodimer of heavy-chains); VHH, the variable domain of the heavy chain of hcAbs consisting of one single-domain, antigen binding fragment (also called as single-domain antibody, sdAb, "nanobody" or "nanoantibody"); ILZ, isoleucine zipper; VN, virus neutralization; HI, hemagglutination inhibition; a-ck, single-domain antibody against cytokeratin 8; HRP, horseradish peroxidase; HA-tag, antigenic determinant, a fragment of 9 amino acids (YPYDVPDYA); PBS, phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4); HMR-analysis, a fingerprinting-like method of parallel restriction analysis of sdAb sequences (using three restriction enzymes – HinfI, MspI, and RsaI).

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with classical types of antibodies in the blood of Camelidae family members and in some chondrichthyan fishes. The existence of an efficient technology for the generation of sdAbs and some useful characteristics of these molecules give them a great deal of potential in immunobiotechnology and medicine. In comparison with conventional Abs, sdAbs are smaller and better able to penetrate tissues and recognize some hidden epitopes that are inaccessible to conventional antibodies. The sdAbs can be obtained by a relatively economical and efficient production method in bacteria and yeast, are highly soluble and resistant to significant changes in temperature and pH, and are simple to use for various genetic engineering approaches (Hamers-Casterman et al., 1993; Greenberg et al., 1996; Nuttall et al., 2001; Harmsen and De Haard, 2007; Wesolowski et al., 2009; Muyldermans et al., 2009; Ghassabeh et al., 2010; Tillib, 2011). These camel antigen-binding domains can be "humanized" without significant loss of their specific activity, via a few amino acid substitutions (Vincke et al., 2009). Therefore, these sdAbs have the potential to be broadly useful for passive immunization to prevent multiple dangerous infections (Wesolowski et al., 2009; Muyldermans et al., 2009; Vanlandschoot et al., 2011).

Starting in 2003 with a collaboration with Prof. S. Muyldermans' laboratory at the Vrije Universiteit in Brussels, Belgium, we have been extensively developing and using the single-domain antibody generation technology for different applications at the Institute of Gene Biology in Moscow, Russia (Rothbauer et al., 2006; Tillib et al., 2010, 2012; Tillib, 2011). The immunization of two-hump camels (*Camelus bactrianus*) with the specific antigen-enriched material is the initial stage of this technology. The immunization step is followed by VHH library generation and a modified phage display-based selection procedure. Typically, a panel of many different sdAb-coding cDNA sequences (clones) is obtained as a result of the procedure. There is a high probability that some of the selected sdAbs will have blocking/neutralizing activity against a particular antigen.

In this study, an avian influenza A virus strain, Mallard/Pennsylvania/10218/84 (H5N2), adapted for mice was used as a model source of antigens for both the immunization and phage display-based selection steps. This virus is lethal for mice and is not dangerous for humans. We obtained a panel of 16 different antiviral (mainly anti-hemagglutinin) sdAb-coding sequences and modified them to produce 'formatted' single-domain antibodies (fsdAbs). Several of these fsdAbs demonstrated neutralizing activity against avian influenza virus *in vitro*. It has previously been shown that the biological activity of initially selected sdAbs can be greatly enhanced by sdAb formatting procedures. For example, the genetic fusion of the coding information of two or more of the neutralizing sdAbs leads to the production of bivalent or multivalent antigen-binding molecules with dramatically increased neutralizing activity both *in vitro* and *in vivo* (Conrath et al., 2001b; Zhang et al., 2004; Coppieters et al., 2006; Roovers et al., 2007; Hmila et al., 2010; Hultberg et al., 2011; Ibañez et al., 2011). Here, we demonstrate that the virus-neutralizing activity of the initially selected antiviral sdAb is significantly increased both *in vitro* and *in vivo* after the formatting procedure involving the addition of two amino acid sequences to the C-terminus of the sdAb: the first, a camel hinge-originated spacer sequence and the second, an isoleucine zipper domain, ILZ (Harbury et al., 1993; Shiraishi et al., 2004).

This is most likely due to the higher avidity and larger size of the fsdAb compared to the sdAb. Intraperitoneal or intranasal administration of the fsdAb at 2 h before or 24 h after viral challenge specifically protects mice from lethal infection with influenza virus.

Passive immunization with a combined preparation of the virus-neutralizing fsdAb and a special recombinant adenovirus expressing the same fsdAb is recommended as an optimal long-acting antiviral remedy. This approach is described in our accompanying paper (Tutykhina et al.) of the journal issue.

2. Materials and methods

2.1. Antigens and camel immunization

An avian influenza virus (H5N2) A/Mallard duck/Pennsylvania/10218/84 (from the collection of the Gamaleya Research Institute for Epidemiology and Microbiology, Moscow, Russia) adapted for mice (Smirnov et al., 2000) was used as a model source of antigen. This virus is lethal for mice and not dangerous for humans. The virus was propagated in the allantoic fluid of chicken embryos at 37 °C for 48 h. Chorioallantoic fluid derived from embryonated eggs infected with an attenuated influenza virus strain A/Mallard duck/Pennsylvania/10218/84 (H5N2) was stored at −70 °C or subjected to ultracentrifugation at 27,000 rpm at 4 °C for 1.5 h. The virus pellet was dissolved in PBS, and the virus-containing fraction was recovered by sucrose gradient centrifugation. Ultracentrifugation was carried out again to remove the sucrose, thereby obtaining a purified virus solution. The viral titer was calculated by titration in MDCK cells using the Reed and Muench method (Reed and Muench, 1938). The 50% lethal dosage (LD50) was calculated by titration in mice. The purified influenza virus was inactivated with glutaric aldehyde for use as an antigen for immunization and ELISA. The glutaric aldehyde treatment was performed by adding glutaric aldehyde to the purified virus solution to a final concentration of 0.1% and leaving the resulting mixture to stand at 4 °C for 1 week.

The full-sized (HA, 525 aa, Cat. No. 11699-V08H, Sino Biotechnological Inc.) and N-terminal portion (HA1, 342 aa, Cat. No. 11699-V08H1, Sino Biotechnological Inc.) of the recombinant hemagglutinin H5 (A/American green-winged teal/California/HKWF609/07) were used for the analyses of the selected and formatted sdAbs.

A two-hump camel (*C. bactrianus*) was immunized (5 subcutaneous immunizations) with inactivated influenza virus (2.5 mg per injection) in Freund's adjuvant (complete for the first injection and incomplete for the other three injections). The second injection was administered 3 weeks after the first, and the remaining three injections were given at 10-day intervals. Blood samples (150 ml) were taken 5 days after the last injection. An equal volume of phosphate buffered saline (PBS) containing heparin (100 U/ml) and EDTA (3 mM) was added to the blood to prevent clotting.

2.2. Construction of a library of sdAb-coding sequences and the selection of sdAbs against avian influenza virus

The DNA cloning (into phagemid vector pHEN4) of the entire repertoire of the variable domains of *Camelidae*-specific heavy chain-only antibodies from peripheral blood B-lymphocytes of an immunized camel was performed according to described procedures (Ghahroudi et al., 1997; Nguyen et al., 2001; Conrath et al., 2001a,b; Saerens et al., 2004).

Single-domain antibodies were selected by panning on an immobilized inactivated influenza virus A/Mallard duck/PA/10218/84 (H5N2). The selection, production of sdAbs in the bacterial periplasm, and the iELISA-based analysis of the ability of the sdAbs to recognize a given antigen were performed using the described techniques (Ghahroudi et al., 1997; Nguyen et al., 2001; Conrath et al., 2001a,b; Saerens et al., 2004), with some modifications (Tillib et al., 2010).

2.3. Selection of sdAbs binding to the avian influenza virus, iELISA

Single-domain antibodies were selected by panning on immobilized inactivated influenza virus A/Mallard duck/PA/10218/84 (H5N2). The selection and production of sdAbs in the bacterial periplasm and the analysis of the sdAb ability to recognize a given antigen were performed using the described techniques (Ghahroudi et al., 1997; Nguyen et al., 2001; Conrath et al., 2001a,b; Saerens et al., 2004) with some modifications (Tillib et al., 2010).

Bacteria from the individual colonies were cultivated and used in parallel to express initially selected sdAbs in the bacterial periplasm as described (Conrath et al., 2001a,b; Saerens et al., 2004). These periplasmic extracts (10 μl) with expressed sdAbs were used in indirect ELISAs (iELISAs) to analyze the specificity and efficiency of sdAb binding to the immobilized influenza virus.

For iELISAs, 10 μg/ml of the A/Mallard duck/Pennsylvania/10218/84 influenza virus or 2 μg/ml of the recombinant proteins (HA or HA1 of influenza virus A/American green-winged teal/California/HKWF609/07 (H5N2)) were passively adsorbed into Nunc Maxisorp 96-well microtiter plates in 100 μl PBS/well overnight at 4 °C. The antigen-coated plates were blocked for 2 h at 37 °C with 1% casein blocking buffer or 1% BSA (Sigma–Aldrich) in PBS. After washing with PBST (1× PBS with 0.1% Tween-20), 10 μl of the sdAb-containing periplasmic fractions or seral dilutions of fsdAbs diluted 1/10 in blocking solution (final volume of 100 μl) were added to the microtiter plates and incubated for 1 h. The binding of the fsdAB was detected with 1/1000 HRP-conjugated anti-HA-tag monoclonal antibodies (CHGT-45P-Z, ICL, Inc., USA) or with 1/5000 anti-camel sdAb rabbit antibody (Tillib et al., unpublished) and then 1/2000 HRP-conjugated anti-rabbit goat antibody (IMTEK, Moscow, Russia). The horseradish peroxidase activity was determined by using ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) as a chromogenic substrate. The absorbance was measured at 405 nm with a plate fluorimeter.

2.4. Subcloning and formatting of initially selected sdAbs sequences and the expression and purification of fsdAbs

The cDNA sequences of the selected sdAbs were subcloned (by conventional or PCR cloning) into the pHEN6 expression plasmid (Conrath et al., 2001a,b) together with the pelB leader sequence (for periplasmic production), the camel upper hinge (the longest hinge variant) and ILZ domain (Harbury et al., 1993) sequences (to obtain the formatted sdAb–fsdAb), and two short tag sequences (HA-tag and $(His)_6$-tag) at the C-terminus coding region as shown in Fig. 2A. The plasmids were transformed into *Escherichia coli* BL21 cells (New England BioLabs) for bacterial expression and purification.

Protein expression was induced by the addition of 1 mM isopropyl-D-1-thiogalactoside (IPTG). After 5–7 h of induction at 37 °C, the cells were harvested by centrifugation and the fsdAbs were purified from the periplasmic extract using Ni–NTA agarose and the QIAExpressionist purification system (QIAGEN). The eluted fraction was concentrated (to a final concentration of approximately 1–5 mg/ml) in Amicon 10 kDa ultrafiltration devices (Millipore) and loaded in volume of 0.5–2 ml onto a Sephacryl S-100 HR 1.5 × 50 gel filtration column (Econo-Column, Bio-Rad). Size-exclusion chromatography was performed using BioLogic LP System (Bio-Rad) at a constant flow rate of 0.3 ml/min with PBS as a running buffer. Void volume of the column evaluated with Blue Dextran 2000 (Sigma–Aldrich) was approximately 35 ml. Fractions were collected according to the obtained UV-absorbance (280 nm) profile and initially verified using polyacrylamide gel electrophoresis (according to Laemmli, 1970). The molecular weight of formatted sdAbs was estimated by comparison of the elution volumes with those of marker proteins with known molecular weight (Bovine albumin, BSA, 67 kDa (Amresco); Ovalbumin, OVA, 45 kDa (Serva); Lysozym, LZ, 14.4 kDa (AppliChem)). Purified (pooled) fsdAbs were again concentrated, affinity purified from endotoxin using Detoxi-Gel Endotoxin Removing Gel (Thermo Scientific), and sterilized by filtration using a cellulose acetate membrane filter (0.2 μm) (Nalgene). The purified fsdAbs were stored in aliquots at 4 °C or, after the addition of 50% glycerol, at −20 °C.

2.5. Hemagglutination inhibition (HI) test

The tests were carried out in U-shaped microtiter plates as described (Smirnov et al., 2000). The sdAb samples were first mixed with chicken erythrocytes to absorb non-specific hemagglutinins. HI titration was then carried out in a microtiter plate with four HA units of influenza virus, 1% chicken erythrocytes and phosphate buffered saline (PBS) as the diluent. The results were read after incubation for 60 min at 4 °C and the titer was defined as the reciprocal value of the last sdAb dilution where inhibition of agglutination was clearly visible.

2.6. VN assay

Madine–Darby canine kidney (MDCK) cells were used in the experiments. The cells were propagated in DMEM supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2.

MDCK cells (2×10^4) were seeded into the wells of a 96-well microtiter plate and incubated at 37 °C in 5% CO_2 overnight. Influenza virus A/Mallard duck/PA/10218/84(H5N2) was added to each sdAb-containing sample at a dose of $100TCD_{50}$. The samples were serially diluted 2-fold. The influenza virus was incubated with the sdAb samples for 15 min at room temperature, and then each sample was added to MDCK cells that had been washed with serum-free DMEM. The cells were incubated for 2 h at 37 °C in 5% CO_2, after which time the serum-free culture media was replased with DMEM containing 5% calf serum, 0.2% BSA and 1 μg/ml trypsin. Incubation of the cultures continued for 3 days, and virus neutralizing titer was then defined.

2.7. Competitive indirect ELISA (CI-ELISA) for affinity evaluation of formatted nanobodies

The CI-ELISAs were performed according to previously described protocols (Bumke and Neri, 2001; Friguet et al., 1985). Maxisorp microtiter plates were coated with 100 μl/well (2 μg/ml) of the full-sized recombinant hemagglutinin HA of influenza virus (A/American green-winged teal/California/HKWF609/07 (H5N2) overnight at 4 °C.

Dry skim milk in PBS (3% MPBS) was used to preblock the wells for 2 h at room temperature. In parallel tubes, fsdAbs at a final concentration of 0.2 nM were incubated with a series of increasing concentrations of HA antigen (in nM: 0, 0.4, 1.23, 3.7, 11, 33, 100, 300 and 900) in PBS for a total reaction volume of 100 μl each. After a 30-min incubation at room temperature, 90 μl of the reaction mixture was combined with 10 μl of 10% MPBS and applied to the coated and preblocked wells of the first of two microtiter plates and incubated for 10 min. Subsequently, the reaction mixtures were transferred to the second antigen-coated microtiter plate, and the ELISA assay was performed as for the first plate. The purpose of the second ELISA assay was to ensure that only a small fraction of the free antibody was captured on the first microtiter plate. The ELISA plates were then washed 6 times in PBS. Bound formatted sdAbs were detected with an HRP-conjugated mouse anti-HA antibody (Santa Cruz Biotechnology), and the horseradish peroxidase activity was measured using ABTS (2,2′-azino-bis(3-ethyl-

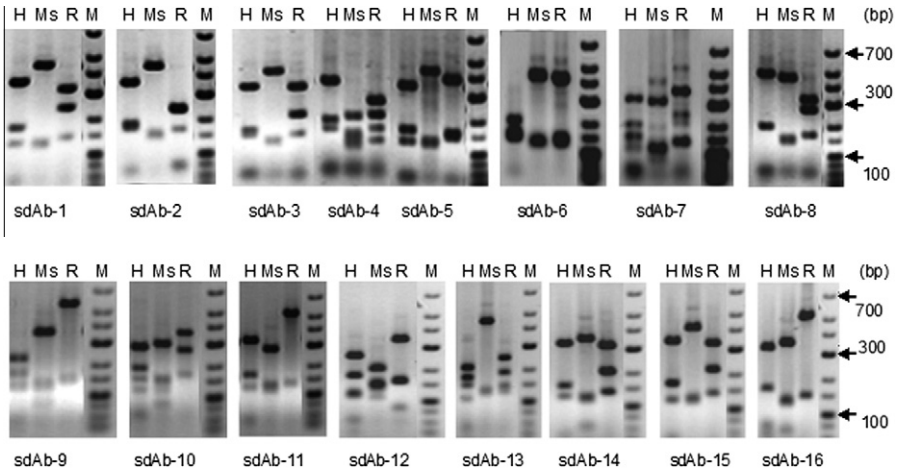

Fig. 1. Fingerprinting-like ('HMR') analysis of selected antiviral sdAb-coding sequences. Each HMR fingerprint is an electrophoretogram consisting of three gel lanes with separated DNA fragments obtained after parallel treatment of the PCR product (an amplified sdAb sequence) with one of three restrictases, HinfI (H), MspI (M) or RsaI (R), and the fourth lane contained marker DNA (the sizes of the marker DNA fragments are shown on the right).

benzothiazoline-6-sulfonate) as the chromogenic substrate. The absorbance was measured at 405 nm with a plate fluorimeter. The concentration of antigen at which the half-maximal ELISA signal is detected corresponds to the dissociation constant Kd. The CI-ELISAs were performed in triplicate.

2.8. Immunization and challenge

Six-week old female BALB/c mice were obtained from the Pushchino Branch of the Institute of the Bioorganic Chemistry, RAS (Pushchino, Russia). The mice were given free access to water and standard rodent chow and were housed in pathogen-free cages. Intranasal infections were performed as described (Lo et al., 2008). Mice with body weights between 18 and 20 g were injected intranasally with 50 $TCID_{50}$ of influenza virus in total volume of 0.01 ml or with concentrated solution (5 mg/ml) of fsdAb. Formatted single-domain antibodies against mouse cytokeratin 8 (a-ck) (Rothbauer et al., 2006) were used as a negative control.

3. Results

3.1. Immunization and selection of a panel of anti-influenza virus (H5N2) single-domain antibodies

One camel (*C. bactrianus*) was immunized with purified influenza virus *A/Mallard duck/Pennsylvania/10218/84* (H5N2). The end-point ELISA titer of *Camelidae*-specific heavy chain-only antibodies (hcAbs) against the virus increased by approximately 80 times (up to 1/7280) in the immune serum compared to the preimmune serum (not shown). RNA was extracted from peripheral blood lymphocytes and converted into complementary DNA. This DNA was used as a template for the PCR cloning of nucleotide sequences coding antigen-binding domains of hcAbs (VHHs, sdAbs) in a phagemid vector to generate a sdAb phage display library as described (Ghahroudi et al., 1997; Nguyen et al., 2001; Conrath et al., 2001a,b; Saerens et al., 2004). Phages carrying influenza virus-specific sdAbs were selected by 2 to 3 cycles of panning on the immobilized virus (Tillib et al., 2010). Individual sequence variants of enriched sdAbs were identified by the fingerprinting-like PCR/restriction analysis (HMR analysis (Tillib et al., 2010)). Fig. 1 shows the HMR fingerprints identifying 16 different variants (groups) of selected sdAbs that recognize the H5N2 influenza virus. The selected sdAb-coding sequences (at least one from each of these groups) were cloned into an *E. coli* expression vector. The corresponding sdAbs (primary sdAbs with an HA-tag and $(His)_6$-tag at their C-terminus) were produced and then purified from the bacterial periplasmic fraction by immobilized metal-affinity chromatography. SdAbs corresponding to all 16 variants shown on Fig. 1 (identified as 'sdAb' with a number) were able to specifically bind immobilized H5N2 influenza virus (data not shown). Unfortunately, these primary (not yet formatted) monovalent sdAbs had weak activities in the *in vitro* hemagglutination inhibition (HI) and virus neutralization (VN) assays. We decided to employ formatting procedures to enhance the biological activities of the selected sdAbs.

3.2. Formatting and characterization of the formatted single-domain antibodies in vitro

We found that an addition of a special linker sequence to the C-terminus of the initially selected (primary) sdAb can be a very reproducible and effective way of formatting. The linker sequence consisted of the spacer sequence (originating from the camel IgG2 upper hinge region) fused with the isoleucine zipper (ILZ) sequence (Harbury et al., 1993; Shiraishi et al., 2004). Presumably, the ILZ sequence is responsible for the posttranslational trimerization of the formatted antibody (Harbury et al., 1993; Shiraishi et al., 2004). Two other peptidic tag sequences (HA-tag and $(His)_6$-tag, HH) were also added to the C-terminal end of the formatted recombinant antibody to improve its detection and purification. The schemes and added sequences of the two types of formatted sdAb structures are shown in Fig. 2A: sdAb-h-HH has the spacer sequence lacking the ILZ domain, whereas the sdAb-h-ILZ-HH has both the spacer and ILZ sequences.

The addition of ILZ domain leads to the effective trimerization of the formatted sdAb. Gel filtration chromatography profiles of differently formatted sdAbs (for initially selected sdAb-7 and sdAb-8) are shown on Fig. 2B to demonstrate this effect. Formatted sdAb-h-HH without ILZ has an expected molecular weight of about 20 kDa, and it is eluted as a single peak with elution volume of about 62–65 ml (between OVA, 45 kDa and LZ, 14.4 kDa). Formatted sdAb-h-ILZ-HH with ILZ has an expected molecular weight of about 23 kDa, and it is eluted (with elution volume of about 40–50 ml) in the close vicinity to the peak of BSA (67 kDa) and clearly earlier than peak of OVA (45 kDa) as it should be in the case of the formatted sdAb trimerization. It can be suggested that possible aggregates or tetramers are eluted before (elution volume of about

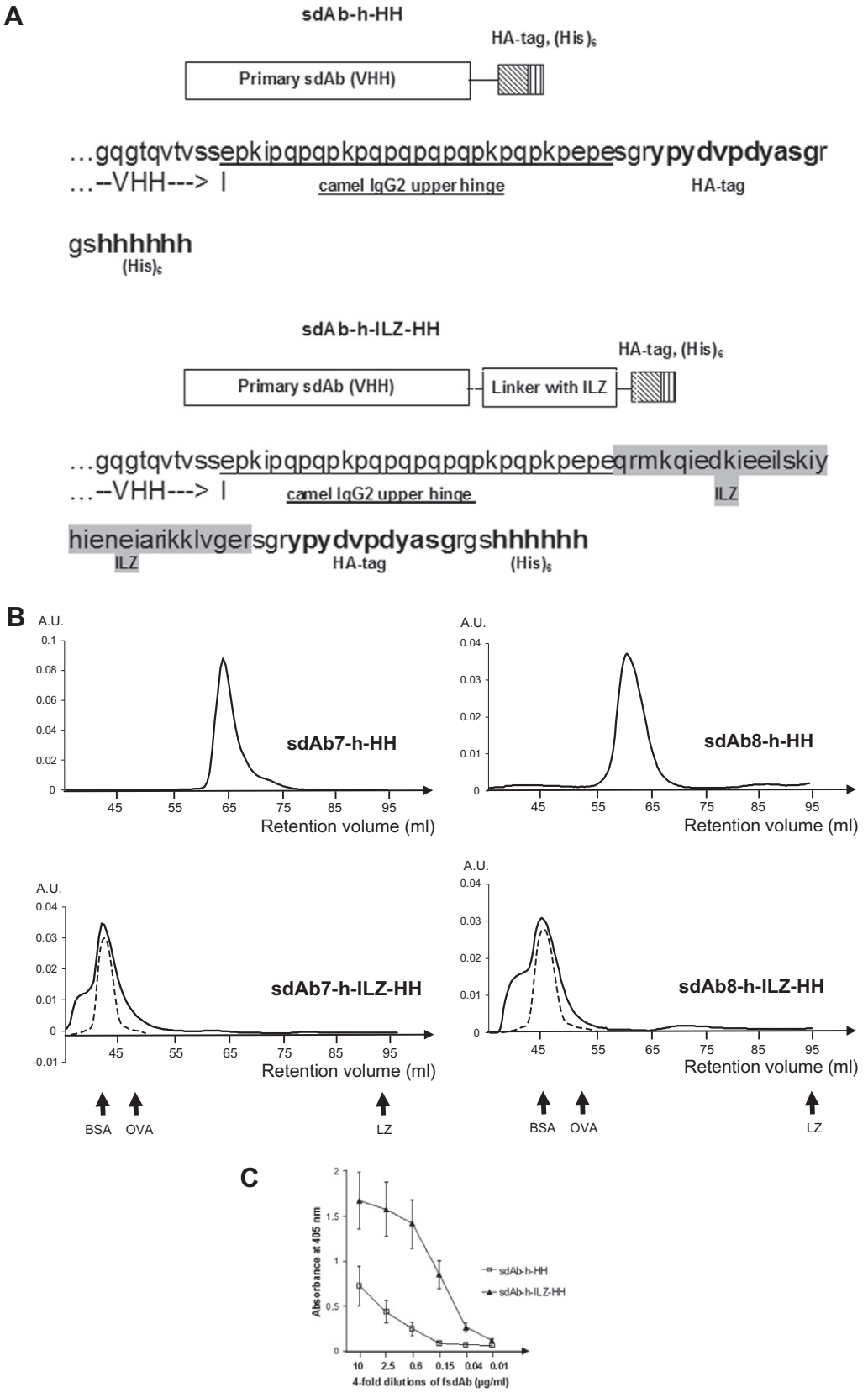

Fig. 2. (A) Schemes and added sequences of two types of formatted sdAb structures: sdAb-h-HH contains the spacer sequence without an ILZ-domain, whereas sdAb-h-ILZ-HH contains both the spacer and ILZ sequences. (B) Gel filtration chromatography profiles of the Ni–NTA eluted periplasmic extracts from *E. coli* expressing differently formatted sdAbs (from initially selected sdAb-7 and sdAb-8), with (sdAb-h-ILZ-HH) or without ILZ domain (sdAb-h-HH), loaded onto a Sephacryl S-100 HR 1.5 × 50 column using PBS as running buffer. Proteins with known molecular weight (Bovine albumin, BSA, 67 kDa; Ovalbumin, OVA, 45 kDa; Lysozym, LZ, 14.4 kDa) were used as markers. (C) Comparative ELISA data of formatted sdAb binding to the immobilized influenza virus demonstrating significant improvement of the avidity upon the addition of the ILZ domain. The results represent the averaged data (with descriptive range error bars) for pairs of the first three different formatted sdAb variants.

Table 1
Hemagglutination inhibition (HI) and the *in vitro* neutralization of influenza H5N2 virus by selected and formatted single-domain antibodies (fsdAbs).

Shortened name of aHAfsdAb	New name of selected and formatted single-domain antibody	Minimal concentration of fsdAb (μg/ml, rough estimate of nM)[a] needed to inhibit hemagglutination	Minimal concentration of fsdAb (μg/ml, rough estimate of nM)[a] needed to neutralize influenza H5N2 virus
aHA-1	aHAfsdAb-1	0.37 μg/ml, 15.7 nM	0.29 μg/ml, 12.3 nM
aHA-2	aHAfsdAb-2	0.10 μg/ml, 4.3 nM	1.40 μg/ml, 59.6 nM
aHA-3	aHAfsdAb-3	1.56 μg/ml, 66.4 nM	0.62 μg/ml, 26.4 nM
aHA-4	aHAfsdAb-4	0.55 μg/ml, 23.4 nM	1.77 μg/ml, 75.3 nM
aHA-5	aHAfsdAb-5	0.70 μg/ml, 29.8 nM	2.20 μg/ml, 93.6 nM
aHA-6	aHAfsdAb-6	0.78 μg/ml, 33 nM	2.50 μg/ml, 106 nM
aHA-7	aHAfsdAb-7	0.24 μg/ml, 10.2 nM	0.10 μg/ml, 4.2 nM
aHA-8	aHAfsdAb-8	0.28 μg/ml, 11.9 nM	0.22 μg/ml, 9.4 nM
aHA-9	aHAfsdAb-9	0.78 μg/ml, 33 nM	0.31 μg/ml, 13.2 nM
aHA-10	aHAfsdAb-10	1.25 μg/ml, 53.2 nM	2.00 μg/ml, 85 nM
aHA-11	aHAfsdAb-11	0.78 μg/ml, 33 nM	0.62 μg/ml, 26.4 nM
aHA-12	aHAfsdAb-12	0.18 μg/ml, 7.66 nM	0.29 μg/ml, 12.3 nM
aHA-13	aHAfsdAb-13	2.30 μg/ml, 98 nM	0.90 μg/ml, 38.3 nM
aHA-14	aHAfsdAb-14	1.17 μg/ml, 49.8 nM	0.90 μg/ml, 38.3 nM
aHA-15	aHAfsdAb-15	0.39 μg/ml, 16.6 nM	0.31 μg/ml, 13.2 nM
aHA-16	aHAfsdAb-16	0.33 μg/ml, 14 nM	1.06 μg/ml, 45 nM

[a] The fsdAb titer (minimal working concentration) was calculated from duplicates as the geometric mean; the concentration was initially calculated for the monomeric fsdAb (average MW of approximately 23,500) and is three times lower when taking the trimerization into account.

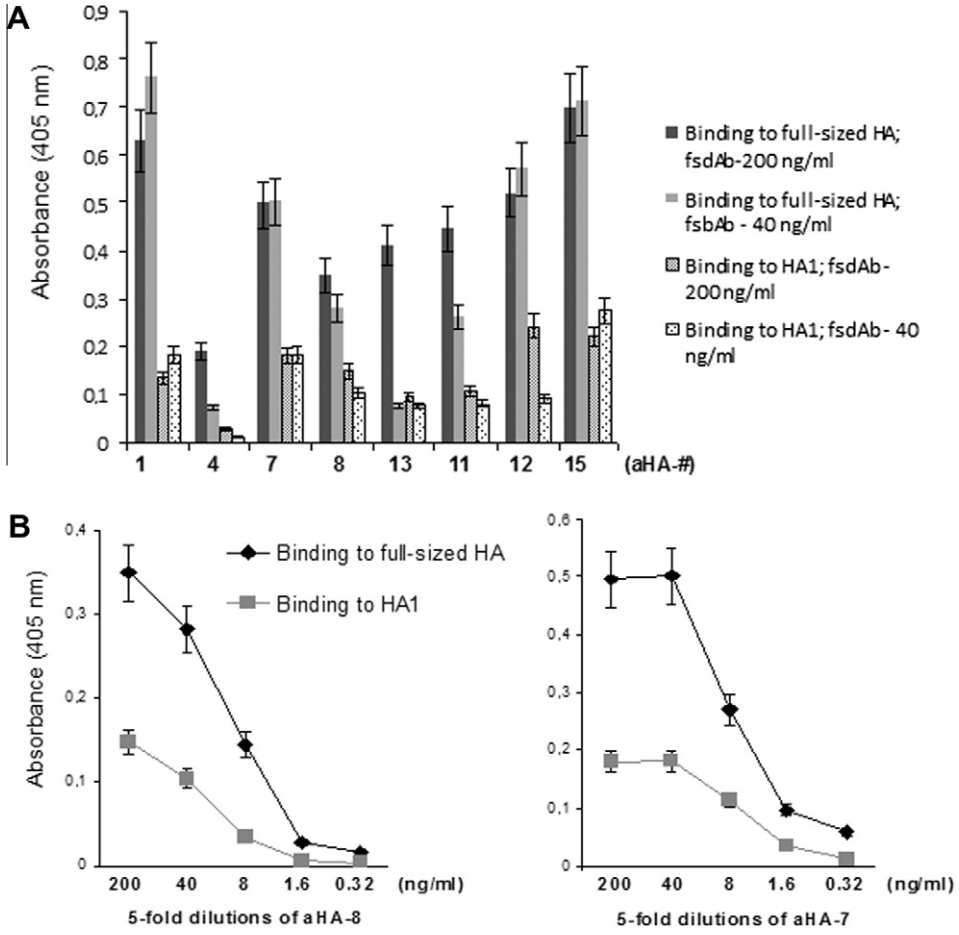

Fig. 3. Indirect ELISA binding data of several fsdAbs to the full-sized recombinant hemagglutinin (HA) of influenza virus (A/American green-winged teal/California/HKWF609/07 (H5N2)), and to its N-terminal domain, HA1 (membrane-distal, globular domain). Both HA and HA1 were immobilized in ELISA plate wells at equal concentrations. (A) ELISA binding data of eight different fsdAbs (aHA-1, aHA-4, aHA-7, aHA-8, aHA-13, aHA-11, aHA-12 and aHA-15, in two concentrations, 40 and 200 ng/ml). (B) ELISA binding data for a series of 5-fold dilutions of aHA-7 and aHA-8. Averaged results of experiments performed in triplicate are shown with descriptive range error bars.

36–42 ml) right before the peak of sdAb-trimer and shoud be separated/removed. The dotted line represents the elution parttern of purified sdAb-h-ILZ-HH.

Comparative ELISA data of the formatted sdAb binding to the immobilized influenza virus (Fig. 2C) show significant improvement of the avidity upon the addition of the ILZ domain. The

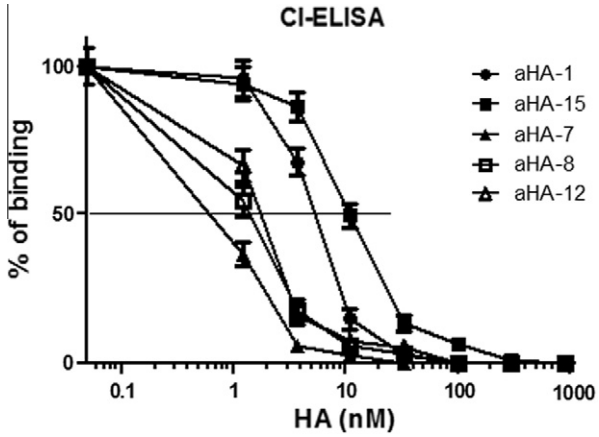

Fig. 4. Competitive indirect ELISA (CI-ELISA) test for the binding of the formatted sdAbs to the recombinant hemagglutinin (HA) of influenza virus (A/American green-winged teal/California/HKWF609/07 (H5N2)). In parallel tubes, fsdAbs (at final concentrations of 0.2 nM) were incubated with a series of increasing concentrations of HA antigen (in nM: 0, 0.4, 1.23, 3.7, 11, 33, 100, 300 and 900) in PBS (total reaction volume of 100 μl each). Bound formatted sdAbs were detected with an HRP-conjugated mouse anti-HA antibody followed by the measurement of the horseradish peroxidase activity using ABTS as a chromogenic substrate. The absorbance was measured at 405 nm with a plate fluorimeter. The maximal ELISA signal corresponds to the 100% binding on the *Y*-axis (absorbance measured for fsdAb in the absence of antigen). The concentration of antigen at which the half-maximal ELISA signal (corresponds to 50% binding) is detected corresponds to the dissociation constant Kd. The CI-ELISA experiments were performed in triplicate. The abscissa (*X*-axis) represents the values (on a logarithmic scale) of the HA antigen concentrations taken for the binding with fsdAb in solution. Averaged results of experiments performed in triplicate are shown with descriptive range error bars.

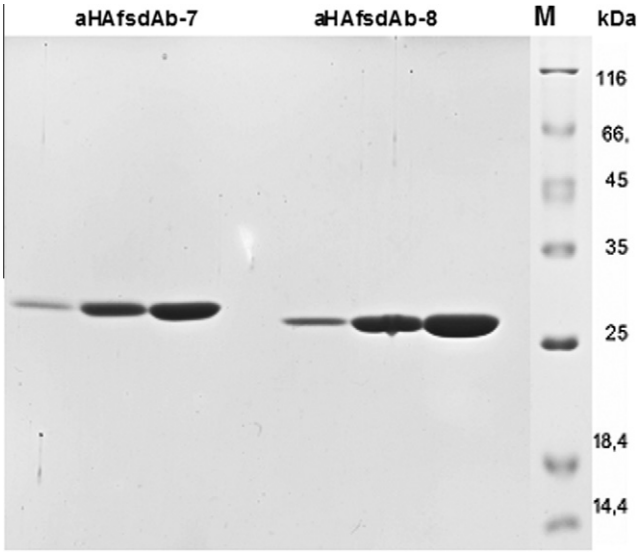

Fig. 5. Purified formatted single-domain antibodies aHA-7 and aHA-8 (for experiments *in vivo*) after fractionation in a 14% SDS–polyacrylamide gel. Three sample lanes correspond to three sequential dilutions for each antibody. Each purified antibody sample was loaded in three sequentional dilutions.

results depicted in Fig. 2C represent the average data for pairs of the first three different formatted sdAb variants. Encouraged by these data, we formatted all other variants of the selected sdAbs into the form of sdAb-h-ILZ-HH (fsdAb) by the same method (molecular cloning). The fsdAbs were produced in the bacterial periplasm and then purified in the same way as the primary sdAbs. The yield of purified fsdAb varied for the different variants (from 0.5 and up to 5 mg from 1 L of bacterial culture) and was generally somewhat lower than that of sdAbs without the ILZ domain (2–10 mg from 1 L of culture). The activities of all fsdAbs were investigated *in vitro* in erythrocyte hemagglutination inhibition (HI) and virus neutralization (VN) assays. The minimal effective fsdAb concentrations (titers) were calculated from the duplicates as geometric means. These data are shown in Table 1. According to both of these assays, the fsdAbs were significantly (approximately 100 times) more active than the corresponding unformatted primary sdAbs. As shown in Table 1 the most active fsdAbs (named as 'aHA' (anti-hemagglutinin) with a number and listed by decreasing activity) are the following: aHA-7, aHA-8, aHA-12, aHA-1, aHA-15, and so on. All of these fsdAbs can bind hemagglutinin, according to the HI assay data. Fig. 3 shows the ELISA results of the binding of several fsdAbs to the full-sized recombinant HA of influenza virus (A/American green-winged teal/California/HKWF609/07 (H5N2)) and to its N-terminal portion, HA1 (membrane-distal, globular domain). Both HA and HA1 were immobilized in ELISA plate wells at equal concentrations. The presented data suggest that the fsdAbs can bind to both proteins, but binding to the full-sized HA is more effective. An easily detectable ELISA signal of the HA binding can be seen (in Fig. 3) in the case of aHA-7 and aHA-8 used at concentrations of 8 ng/ml (approximately 0.2 nM for trimeric fsdAbs). Given the high sensitivity of this ELISA, we decided to perform competitive indirect ELISAs (CI-ELISAs) to evaluate the fsdAb-influenza virus binding affinity constants and to compare the affinities and avidities of the most promising fsdAbs obtained.

CI-ELISAs were performed according to previously described protocols (Bumke and Neri, 2001; Friguet et al., 1985). This assay allows the determination of the affinity constants of antibodies in solution with negligible avidity effects. The concentration of the antibody should be as low as possible, close to or lower than the Kd value. The fsdAbs were used at a concentration of approximately 0.2 nM (based on the calculation for the trimeric fsdAb). Full-sized recombinant avian HA was used as the antigen. The low amount of antibody was incubated with increasing concentrations of antigen in parallel reactions in an aqueous solution. When the binding equilibrium is established, the fraction of unbound antibody can be captured on an antigen-coated microtiter plate and can be detected by a conventional (indirect) ELISA procedure. We used commercial mouse monoclonal antibodies specific for the HA tag (horseradish peroxidase-conjugated) as the secondary antibody to detect the bound fsdAb. This peptidic HA-tag region (presented in the fsdAb) is missing and not recognized by ELISA in the case of the recombinant bird hemagglutinins (HA and HA1) used in this study (data not shown). At equilibrium, the dissociation constant Kd can be defined as $Kd = [A][B]/[AB]$, where [A], [B] and [AB] are concentrations of the free antibody, free antigen and antibody–antigen complex, respectively. At semisaturation (when $[A] = [AB]$), the dissociation constant Kd should be approximately equal to the free antigen concentration ($Kd = [B]$,) and approximately equal to the total concentration of antigen ($Kd \approx [B]_{total}$) if to take into account the low concentration of antibody ($[A]_{total} \ll Kd$). In the CI-ELISA, the highest signal (corresponding to the amount of fsdAb bound to the immobilized HA antigen in the microtiter plate) is observed in the absence of the HA antigen, and the lowest signal is observed at high concentrations of antigen. The concentration of antigen at which the half-maximal ELISA signal is detected should correspond to the Kd (Bumke and Neri, 2001]. The results of the CI-ELISAs for five fsdAbs (with the highest activities in the HI and VN assays shown in Table 1) are shown in Fig. 4. Maximal ELISA signal corresponds to the binding of 100% on the *Y*-axis (absorbance measured for fsdAb in the absence of antigen). Half-maximal ELISA signal corresponds to 50% of binding. The abscissa (*X*-axis) represents values (on a logarithmic scale) of HA antigen concentrations taken for binding with fsdAb in solution.

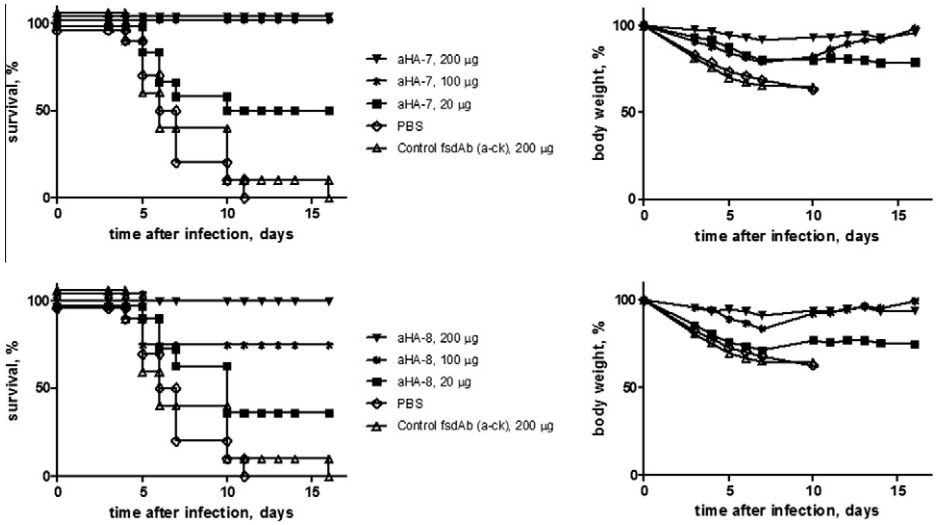

Fig. 6. Intraperitoneal injection of aHAfsdAb-7 (aHA-7, data shown at the upper part of the figure) or aHAfsdAb-8 (aHA-8, data shown at the bottom part of the figure) 2 h before infection protects mice from H5N2 influenza virus.

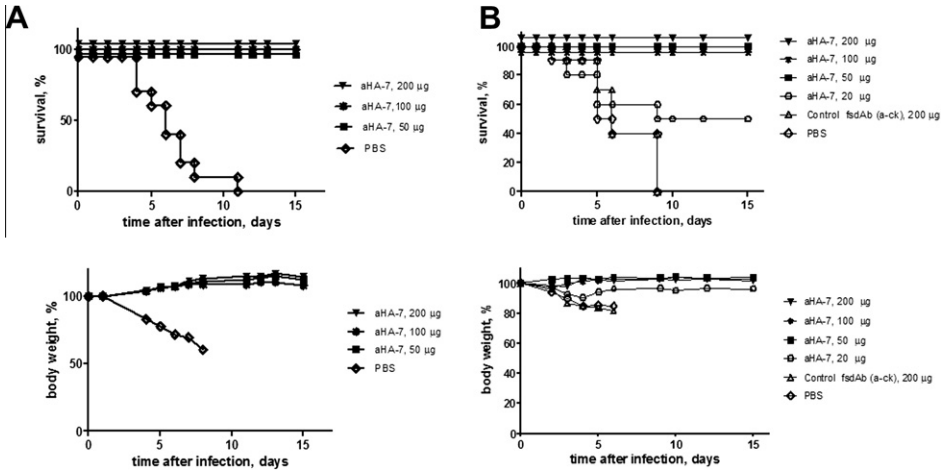

Fig. 7. Intranasal injections of aHAfsdAb-7 (aHA-7) 2 h before infection (A) or 24 h after infection (B) protect mice from H5N2 influenza virus.

All investigated formatted sdAbs had estimated Kd in the low nM range (from 0.7 nM for aHA-7 and 1.3 nM for aHA-8 up to 10.5 nM for aHA-15). We suggest that the significant improvement in the "experimental affinity" in the case of trivalent versus monovalent fsdAb can be attributed mainly to the increased antibody avidity. FsdAbs aHA-7 and aHA-8 showed the best properties in all of our tests *in vitro*. These two fsdAbs were chosen for the further evaluation of *in vivo* virus-neutralization activities.

3.3. Demonstration of anti-viral neutralization activity of selected formatted single-domain antibodies in vivo

Formatted sdAbs aHA-7 and aHA-8 (in the form of VHH-h-ILZ-HH) were expressed in and initially purified from the bacterial periplasm. The sdAbs were then dialyzed against PBS and additionally purified from endotoxin by passing through columns with Detoxi-Gel Endotoxin Removing Gel (Thermo Fisher Scientific Inc.). Fig. 5 shows the fsdAbs aHA-7 and aHA-8 after purification and subsequent separation on polyacrylamide gel (according to Laemmli, 1970). Practically all of the purified protein in both cases formed one major band with a size of approximately 27 kDa.

The protective efficacy of these purified anti-influenza HA (H5N2) fsdAbs were evaluated *in vivo*. The fsdAbs, at 20, 100 or 200 μg per mouse, were administered intraperitoneally to Balb/c mice 2 h prior to challenge with a lethal dose (50 LD50) of influenza virus A/Mallard duck/Pennsylvania/10218/84 (H5N2). The survival and body weight changes (in %) were monitored for 16 days after infection, and the results are presented in Fig. 6. FsdAb aHA-7 demonstrated total (100%) protection at doses of 100 and 200 μg per mouse. At a dose of 20 μg per mouse, aHA-7 yielded 50% protection, and 100% mortality was observed in control groups of mice injected with PBS or with an anti-cytokeratin (a-ck) fsdAb. FsdAb aHA-8 gave a slightly lower degree of protection; all of the mice were protected at a dose of 200 μg per mouse, and doses of 100 and 20 μg per mouse protected 70% and 40% of the mice, respectively.

Finally, the *in vivo* protective activity of fsdAb aHA-7 was investigated using intranasal immunization of mice 2 h before and 24 h after challenge with a lethal dose (50 LD50) of influenza virus A/Mallard duck/Pennsylvania/10218/84 (H5N2). Strong protection was observed for fsdAb doses above 50 μg per mouse administered intranasally 2 h before viral infection (Fig. 7A). These data were similar to those described above for intraperitoneal immunization.

The therapeutic efficacy of fsdAb aHA-7 are presented in Fig. 7B, and these data appear promising. The intranasal injection of the fsdAb can effectively protect mice even 24 h after challenge with a lethal dose (50 LD50) of influenza virus. In this case, 100% survival was observed at fsdAb aHA-7 doses of 50, 100 and 200 μg per mouse.

4. Discussion

This work continues a series of recently published studies that employ recombinant single-domain antibody (sdAb, or nanobody®) generation technologies to battle viruses by a passive immunization approach (Hultberg et al., 2011; Ibañez et al., 2011; Vanlandschoot et al., 2011). Here, we describe (as a proof of principle) a modified approach to efficiently generate protective molecules against newly emerging strains of influenza virus or potentially other types of virus within a reasonably short period of time (within a few months). An avian influenza virus A/Mallard/Pennsylvania/10218/84 (H5N2) strain adapted for mice was used as a model source of antigen for both the immunization and phage display-based selection procedures.

Our approach includes the following principal steps.

First, the immunization of a two-hump camel (*C. bactrianus*) is performed with the specified antigen-enriched material (here, a particular strain of inactivated influenza virus). Our previous experience, as well as reports from other researchers suggested that the immunization step is typically the most reliable way to obtain high-affinity sdAbs. However, it is also important for the antigen to be presented in as natural a form as possible. SdAbs often recognize conformational epitopes such as clefts or pockets, and sdAbs are unique in their ability to recognize uncommon or hidden (for classical types of Abs) epitopes (Conrath et al., 2001a; Stijlemans et al., 2004; De Genst et al., 2006; Vanlandschoot et al., 2011). Therefore, in contrast to previous works, we used inactivated viral particles as the antigenic material. Previously, we have successfully used the total *Drosophila* embryonic nuclear extract to select anti-lamin sdAb (Rothbauer et al., 2006), and others have demonstrated the feasibility of using complex proteomic mixtures, rather than purified protein antigens, to obtain high-quality sdAb (Saerens et al., 2008).

Second, some new modifications in the panning procedure are suggested, including the use of antigenic material in the most natural form possible (phage particles). Our previous experience in the panning technique allowed us to make some important improvements in the selection procedure, including the use of different blocking solutions at subsequent selection steps, the parallel use of two different helper phages, and 'HMR-analysis' or sequencing of all selected sdAb variants at each stage of selection (regardless of their degree of enrichment) (Tillib et al., 2010).

Third, a new type of sdAb formatting is proposed. Here, the initially selected monovalent sdAbs had weak biological activities as is usually the case. It was shown that the sdAb activity can be greatly enhanced by sdAb formatting procedures (Conrath et al., 2001b; Zhang et al., 2004; Coppieters et al., 2006; Roovers et al., 2007; Hmila et al., 2010; Hultberg et al., 2011; Ibañez et al., 2011). Unfortunately, straightforward cloning of two sdAb copies separated by the Gly/Ser-rich spacer sequence in one expression plasmid vector was not reliable in our hands (in the case of different *E. coli* strains) and too often resulted in slight, unpredictable mutations in the sdAb sequences. These mutations represent a significant problem, given the large number of selected sdAb variants. Based on the analysis of published data, we decided to use another method of sdAb formatting, which involves the addition of a special type of coiled-coil sequence (the isoleucine zipper domain, or ILZ (Harbury et al., 1993; Shiraishi et al., 2004)) separated by a camel hinge-originated spacer sequence from the sdAb sequence to the C-terminus of the sdAb. This ILZ is a mutated version of the well known leucine zipper domain, which is located at the C-terminus of the yeast transcriptional activator protein GCN4 and is responsible for the formation of a very stable coiled-coil dimer of alpha helices with a parallel orientation (O'Shea et al., 1989). Presumably, the ILZ domain containing peptides adopt trimeric parallel conformations (Harbury et al., 1993; Sorger and Nelson, 1989), which could lead to a significant increase in the biological activities of these peptides (Sorger and Nelson, 1989; Shiraishi et al., 2004). The resulting trimeric (trivalent) antibody may be particularly suitable in the case of hemagglutinin binding/blocking, as it has been shown that the hemagglutinin glycoprotein of influenza virus is a trimer (Wilson et al., 1981).

After the formatting, the biological activities of our initially selected anti-influenza virus (H5N2) sdAbs were significantly increased. It was demonstrated that one intranasal injection of 50 μg or more of the fsdAb aHA-7 can effectively protect mice even 24 h after challenge with a lethal dose (50LD50) of influenza virus.

It was shown previously that such sdAb molecules could be further adapted for more efficient action within body fluids. They can be humanized and connected to special protein domains to extend the time spent circulating in the blood without a loss in functionality. Thus, the described approach of sdAb generation, selection and formatting can efficiently lead to the production of protective molecules against particular, newly emerging strains of influenza within a reasonably short period of time. Concerning the issue of the high genetic variability of the HA glycoprotein, recent data from several laboratories report the existence of highly conserved epitopes located mainly on the stem region of the HA or in other regions. Antibodies specific for these epitopes are able to neutralize a broad spectrum of influenza viruses belonging to different subtypes (Sui et al., 2009; Yamashita et al., 2010; Whittle et al., 2011; Krause et al., 2011; Corti et al., 2011; Ekiert et al., 2011; Kaminski and Lee, 2011; De Marco et al., 2012). Therefore, our approach combined with a selection focused on these conservative epitopes should lead to the production of fsdAb-based molecules protective against a broad spectrum of influenza virus subtypes.

Acknowledgments

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