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Human monoclonal antibodies in single chain fragment variable format with potent neutralization activity against influenza virus H5N1

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ABSTRACT

Effective diagnostic and therapeutic strategies are needed to control and combat the highly pathogenic avian influenza virus (AIV) subtype H5N1. To this end, we developed human monoclonal antibodies (mAbs) in single chain fragment variable (scFv) format towards the H5N1 avian influenza virus to gain new insights for the development of immunotherapy against human cases of H5N1. Using a biopanning based approach a large array of scFvs against H5N1 virus were isolated from the human semi-synthetic ETH-2 phage antibody library. H5N1 ELISA-positive scFvs with unique variable heavy (VH) and light (VL) chain gene sequences showed different biochemical properties and neutralization activity across H5N1 viral strains. In particular, the scFv clones AV.D1 and AV.C4 exerted a significant inhibition of the H5N1 A/Vietnam/1194/2004 virus infection in a pseudotype-based neutralization assay. Interestingly, these two scFvs displayed a cross-clade neutralizing activity versus A/whooping swan/Mongolia/244/2005 and A/Indonesia/5/2005 strains. These studies provide proof of the concept that human mAbs in scFv format with well-defined H5N1 recognition patterns and in vitro neutralizing activity can be easily and rapidly isolated by biopanning selection of an entirely artificial antibody repertoire using inactivated H5N1 virus as a bait.

1. Introduction

The highly pathogenic influenza A (H5N1) virus causes severe disease in humans and poses an unprecedented pandemic threat (Li et al., 2004; Ungchusak et al., 2005). The antiviral agents such as amantadine and rimantadine or neuraminidase inhibitor oseltamivir constitute an important treatment option. However, pharmacological treatment is often associated with the selection of drug resistant virus strains (Li et al., 2004; Puthavathana et al., 2005) and oseltamivir-resistant H5N1 variants are isolated during oseltamivir treatment (de Jong et al., 2005; Le et al., 2005). Currently, there are a number of obstacles for the development of safe and effective pandemic influenza vaccines (Hoelscher et al., 2008; Tambyah, 2008). Surviving patients who have recovered from H5N1 infection may have neutralizing antibodies, thus suggesting that antibody-mediated immunity may contribute to eliminating infection (de Jong et al., 2006). The advantages of human antibodies, particularly their lack of immunogenicity, make them very attractive for therapeutic applications. Fully human mAbs with neutralizing H5N1 activity were generated from Epstein Barr virus (EBV)-immortalized memory B cells of infected donors (Simmons et al., 2007) or they were genetically engineered and transiently expressed in mammalian recipient host cells (Hanson et al., 2006; Kashyap et al., 2008). These findings highlight the potential of immunotherapy as a viable treatment option in human cases of avian influenza. However, influenza viruses rapidly mutate, particularly in the regions of hemagglutinin (HA) responsible for antigenicity (Abdel-Ghafar et al., 2008), suggesting that the development of a useful passive immunization strategy should include simple and rapid procedures to promptly respond to the threat of an influenza pandemic.

Phage antibody technology appears to offer option, as human antibody fragments can be isolated from repertoires of fragments displayed on filamentous bacteriophage (Hoogenboom, 2005; Benhar, 2007). The process does not require immunization of humans or the handling of unsafe cells or antigens. The fragments are secreted into the bacterial periplasm and culture medium and can be produced on a large scale in a prokaryotic system, so

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reducing costs and time spent on safe and effective drug substance production.

2. Materials and methods

2.1. Antigens

The H5N1 virus used in this study is a β-propiolactone (BPL) inactivated, purified H5N1 A/Vietnam/1194/2004 virus strain (NIBRG-14) provided by the National Institute for Biological Standards and Control (NIBSC). NIBRG-14 was developed at NIBSC by using a "reverse genetics" approach with the hemagglutinin (HA) and neuraminidase (NA) genome segments derived from A/Vietnam/1194/2004 (H5N1) and the remaining six segments from an A/Puerto Rico/8/34 (H1N1) background (Nicolson et al., 2005). This reverse genetically modified reassortant of H5N1 will be hereinafter termed H5N1/NIBRG-14. H5N2 (A/Mallard/Italy/80/1993), H5N3 (A/Mallard/Italy/208/2000) and H1N1 (A/Mallard/Italy/185/1996) live avian influenza (AIV) virus strains were a generous gift of Dr. Delogu (Department of Public Health and Animal Pathology, Faculty of Veterinary Medicine, University of Bologna, Italy). Inactivated A/Solomon Islands/3/2006 (H1N1) and A/Wisconsin/67/2005 (H3N2) are the main active ingredients of Mutagrip Pasteur vaccine (Sanofi Pasteur, Lyon, France).

Influenza lentiviral pseudotypes used in this study are lentiviral particles engineered to express both the luciferase reporter gene and the heterologous HA protein from H5N1 viruses on their surface (Sanders, 2002; Temperton et al., 2007). These lentiviral vectors were used as surrogate viruses for studying neutralizing antibody responses to viral infection in the neutralization assays that we conducted.

2.2. ETH-2 antibody phage library

The semi-synthetic human recombinant antibodies library ETH-2 (Viti et al., 2000) consisting of a large array (more than 10^9 antibody combination) of scFv polypeptides displayed on the surface of M13 phage was used for the isolation of H5N1-specific antibodies. The library was built up by means of random mutagenesis of the Complementary Determining Region 3 (CDR3) of only three antibody germline gene segments (DP47 for the VH chain, DPK22 and DPL16 for the VL chain). Diversity of the VH chain was created by randomizing four to six positions which replaced the pre-existing positions number 95–98 of the CDR3. The diversity of the VL chain was created by randomizing six positions (91–96) in the CDR3. Antibody residues are numbered according to Tomlinson et al. (1995).

2.3. Isolation of phage antibodies from ETH-2 library

A panel of scFvs against H5N1 were obtained by an affinity enrichment process commonly known as *biopanning* consisting of the absorption and elution of a phage antibody library to target antigen immobilized on immunotubes. In brief, phage antibodies are captured by the target antigen during incubation and then unbound phages are washed away from the solid surface. By repeating this cycle several times using phage antibodies previously recovered from the solid surface by pH treatment, only antigen-specific phage antibodies are isolated. For selection of H5N1 specific phage antibodies an aliquot of the ETH-2 library, containing 10^{13} colony forming units (cfu) phage was adsorbed in 4 ml phosphate-buffered saline (PBS) to the immunotubes (Nunc Maxisorp; Denmark) coated overnight (ON) at room temperature (RT) with 5 μg/ml of inactivated, purified H5N1/NIBRG-14 virus in PBS. After *biopanning*, phages were eluted with 1 ml of 100 mM triethylamine and the solution was immediately neutralized by adding 0.5 ml of 1 M Tris–HCl pH 7.4. Eluted phages were used to infect log phase TG1 *E. coli* bacteria [supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proAB+ lacIqlacZΔM15)] and amplified for the next round of selection as described elsewhere (Flego et al., 2005; Viti et al., 2000). Briefly, 50 ml of 2 × YT medium (Sigma–Aldrich, St. Louis, MO) with 100 μg/ml ampicillin (2 × YTA) and glucose 1% were inoculated with enough bacterial suspension to yield an optical density $(OD)_{600nm}$ of 0.05–0.1. The culture was grown up to OD_{600nm} of 0.4–0.5 and infected with M13 K07 helper phage at a ratio of around 20:1 phage/bacteria. The rescued phages were concentrated by precipitation with polyethylene glycol (PEG) 6000 and used for subsequent rounds of panning (usually three rounds to recover antigen-specific antibody phages from the ETH-2 library). Plating on agar of TG1 cells infected with a pool of phage antibodies from third selection allowed individual clones harbouring phagemid to grow. Several bacterial clones were tested for their ability to secrete functional, anti-H5N1 scFvs. For soluble scFv preparation, the individual colonies were grown in 96 flat bottomed wells (Nunc) for 2 h at 37 °C in 180 μl 2 × YTA medium and glucose 0.1% in 96-well plates and induced with 50 μl 2 × YTA medium and 6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma–Aldrich). The following day the plates were spun down at 1800 g for 10 min and the supernatants containing soluble scFvs were recovered and tested for specificity.

2.4. ELISA

96-well ELISA-plates (Nunc) were coated ON at RT with 0.5 μg of antigen (virus or irrelevant antigen) in PBS. The following day a blocking solution, consisting of 2% non-fat dry milk in PBS (MPBS), was added; plates were washed with PBS containing 0.05% Tween 20 (TPBS) and incubated for 2 h at RT with 50 μl of supernatants containing soluble scFv antibody, anti-Flag M2 antibody (1.6 μg/ml; Sigma–Aldrich) and anti-mouse HRP conjugated antibody (1.6 μg/ml; Dako, Denmark). The reaction was developed using 3.31–5.51-tetramethylbenzidin BM blue, POD-substrate soluble (Roche Diagnostics; Indianapolis, IN) and stopped by adding 50 μl of 1 M sulfhydric acid. The reaction was detected with an ELISA reader (Biorad, Hercules, CA), and the results were expressed as A (absorbance) = $A(450\,nm) - A(620\,nm)$. In ELISA for screening of H5N1 specific phage antibodies the wells equal to and three time more than background signal were considered as positive.

2.5. DNA characterization and sequences

Plasmid DNA from individual bacterial colonies was digested with specific endonucleases; CDR (complementarity determining regions) were sequenced with an automated DNA sequencer (M-Medical/Genenco, Pomezia, Italy) using Fdseq1 (5′-GAA TTT TCT GTA TGA GG-3′) and pelBback (5′-AGC CGC TGG ATT GTT ATT AC-3′) primers.

2.6. Western blot

1 μg of inactivated, purified H5N1/NIBRG-14 virus or irrelevant protein was loaded onto 10% SDS-PAGE in reducing and non-reducing conditions, and then transferred to a nitrocellulose membrane using standard procedures. The membrane was blocked in 4% MPBS ON at RT. Blotted proteins were incubated for 2 h with supernatant containing soluble scFvs, washed with TPBS and incubated again with 5 μg/ml anti-Flag M2 mouse antibody (Sigma–Aldrich) in 2% MPBS. After an additional incubation for 1 h at RT in presence of 5 μg/ml of a goat anti-mouse antibody HRP-conjugated (Dako), the reaction was developed and visualized with a chemiluminescence detection kit (Pierce, Rockford, IL). Monospe-

cific rabbit immunoserum (ProSci Inc., Poway, CA) raised against a peptide corresponding to a conserved sequence from HA1 region of H5N1 was used as positive control.

2.7. Soluble ScFv purification

The positive clones were cultured for large scale scFv production. TG1 *E. coli* infected cells were cultured at 30 °C in 2 × YT containing 100 μg/ml ampicillin and 0.1% glucose till OD_{600nm} of 0.5. After induction of antibody expression by adding 1 mM IPTG to culture, cells were incubated for another 3 h at 30 °C. Periplasmic extracts were prepared by resuspending the bacterial pellet in 1/20 the original volume of 30 mM Tris, pH 7.0, 20% sucrose and 1 mM EDTA. After incubation for 20 min on ice, the debris was removed by centrifugation and supernatants (periplasmic fraction) were filtered (0.2 μm, Millipore, Bedford, MA). His-tagged scFv fragments were purified by immobilized metal affinity chromatography using Ni2± nitriloacetic acid agarose (Qiagen, Madison, WI). ScFv fragments were eluted with 250 mM imidazole in PBS, dialyzed against PBS, tested for specific antigen recognition and stored at −80 °C in small aliquots.

2.8. Hemagglutination-inhibition (HI) test

ScFv fragments were tested for antibodies to the influenza H5N1/NIBRG-14 virus strain by the HI test. The HI test was performed using horse red blood cells (HRBCs) according to standard procedures with minor modifications (Stephenson et al., 2003). Basically, a 1% suspension of HRBCs in 0.5% BSA/PBS was used throughout the procedure and HI titres were read after 60 min.

2.9. Generation of pseudotypes bearing H5 hemagglutinin

Influenza lentiviral pseudotypes, expressing luciferase reporter gene and bearing HA protein on the surface, were generated as previously described (Temperton et al., 2007). In brief, 293T cells were co-transected with the following four plasmids: the gag-pol and rev constructs, the pI.18 plasmid expressing HA from influenza A/Vietnam/1194/2004 (H5N1, clade 1), and the reporter plasmid expressing renilla luciferase gene (Naldini, 1998; Temperton et al., 2007). In order to obtain pseudotypes bearing HA from H5N1 clade 2 strains, nucleotide sequences encoding A/Indonesia/5/2005, A/whooping swan/Mongolia/244/2005 and A/Anhui/1/2005 HAs were synthesized by GeneArt (Regensburg, Germany). HA sequences were subsequently cloned into the suitable pI.18 vector. The multi-basic cleavage site of A/Vietnam/1194/2004 HA was substituted into the other HA sequences in an effort to generate functionally active pseudotyped vectors and to confer similar entry (Kong et al., 2006). Twenty-four hours post infection, 1 U of exogenous neuraminidase (Sigma–Aldrich) was added to allow the release of pseudotypes. Culture supernatants were collected 48 and 72 h post-infection, filtered and stored at −80 °C. Pseudotypes were then titrated on 293T cells and a corresponding amount of pseudotypes giving RLU values of 100–150 was used in the neutralization assay.

2.10. Neutralization assay

Neutralizing antibodies were measured as reductions in luciferase reporter gene expression after a single round of pseudotypes infection in 293T cells as recently described (Temperton et al., 2007). Briefly, 200 pseudotypes giving 100–150 Relative Luminescence Unit (RLU) were incubated with various dilutions of sheep serum or scFv at various dilutions for 1 h at 37 °C in 96-well flat-bottom culture plates. Freshly trypsinized cells were added to each well. After 48 h incubation, cells were lysed and transferred to 96-well white solid plates for measurements of RLUs values by using a Molecular Devices Luminometer. The 50% inhibitory dose (ID_{50}) was defined as the sample concentration that caused a 50% reduction in RLU compared to virus control wells after subtraction of background RLU.

Immune sheep sera against H5N1/NIBRG-14 were obtained by NIBSC.

3. Results

3.1. ScFv antibodies to H5N1

To isolate specific scFv antibodies, an aliquot of the human semi-synthetic ETH-2 library containing about 1×10^{13} cfu phages was introduced for panning into immunotubes coated with inactivated, purified H5N1/NIBRG-14 virus as a bait. Non-specifically absorbed phages were removed by washing. Bound phages were eluted, amplified and used for further rounds of panning as described (Flego et al., 2005; Viti et al., 2000). After three rounds of selection phage antibody populations specifically recognizing the H5N1/NIBRG-14 virus were isolated. Plating on agar TG1 phage antibody-infected cells allowed the growth of individual phagemid clones. Soluble scFvs derived from IPTG induced colonies, were screened by ELISA and several of them proved to be specific for H5N1/NIBRG-14 virus (Fig. 1). Molecular studies showed that all scFv H5N1-positive antibodies had a MW of about 27 kDa as expected from the biochemical constitution of these immunoglobulin (Ig) fragments (Viti et al., 2000) and that the genes encoding for VH and VL chains displayed on M13 phage were correctly expressed (data not shown).

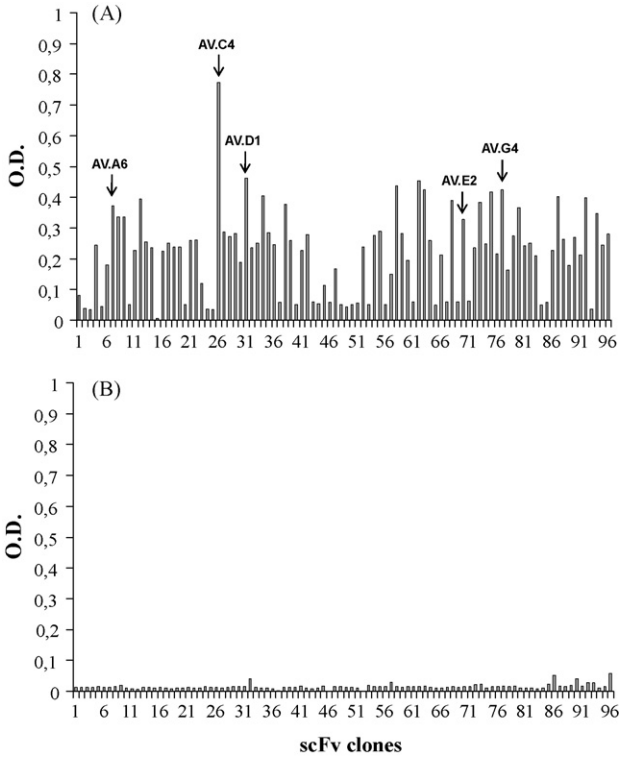

Fig. 1. Screening of H5N1-specific scFv clones. IPTG-induced bacterial supernatants of individual colonies from the third round of the ETH-2 library selection were assayed for H5N1 specificity. H5N1/NIBRG-14 virus or irrelevant antigen (glucose oxidase) coated 96-well microtiter plates were tested by ELISA. OD values of the scFv clones against inactivated, purified H5N1/NIBRG-14 virus (panel A) or irrelevant antigen (panel B) are shown. The most ELISA reactive scFv clones chosen for further studies are arrowed.

VH CHAIN (CDR3) DP 47 gene						VL CHAIN (CDR3) DPL16 gene						
95(*)					99	91					96	
P	T	H	A	G	T	P	P	L	P	V	Y	**AV.A6**
ccg	act	cat	gcg	ggt	act	ccc	cct	ctg	cct	gtg	tat	
I	V	P	K	R	V	Q	P	P	F	G	S	**AV.C4**
att	gtg	ccg	aag	cgg	gtt	cag	ccg	ccc	ttt	ggt	agt	
H	G	T	R	R	A	E	P	P	M	A	G	**AV.E2**
cat	ggt	acg	cgg	cgt	gct	gag	ccc	ccg	atg	gct	ggg	
R	Q	T	L	A		P	L	P	D	I	G	**AV.G4**
agg	cag	acg	ctt	gct		ccg	ctg	ccc	gat	att	ggt	
R	K	R	G			P	S	P	L	L	G	**AV.D1**
cgg	aag	cgt	ggt			ccc	tcg	ccg	cct	cct	ggg	

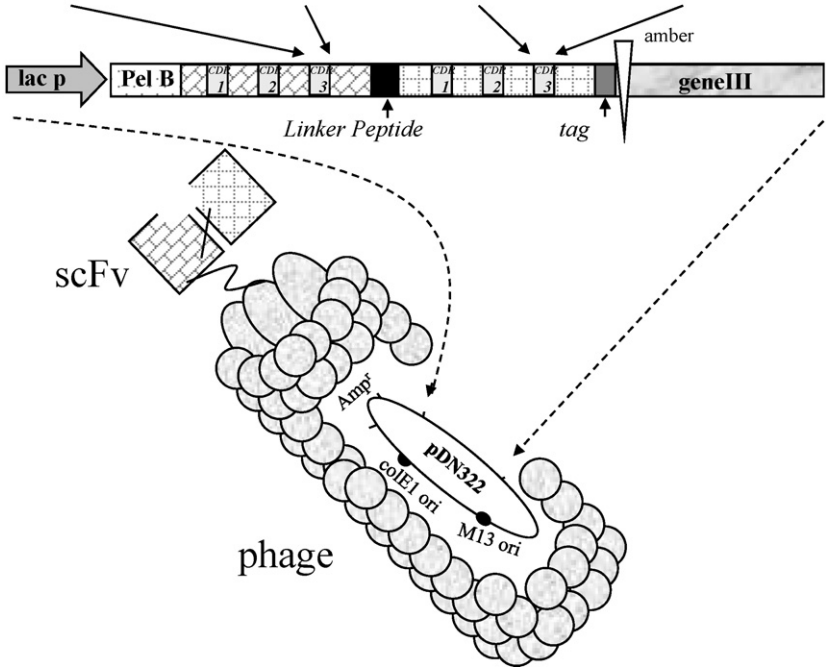

Fig. 2. Gene encoding for scFv antibodies. In panel A, the amino acid sequences of VH and VL chains in a single letter code and their residue positions in the CDR3 regions are reported. In panel B, the schematic representation of the scFv antibody displayed on M13 phage as pIII fusion proteins is depicted. Single amino acid codes are used according to standard IUPAC nomenclature. (*)Numbering is according to Tomlinson et al. (1995).

3.2. Specificity of the scFv antibodies for H5 subtype of HA

Five of the most ELISA reactive scFv clones, each one representative of a distinct class of antibody according to VH and VL chain gene sequences (Fig. 2), were tested on different AIVs (Table 1). While none of the five scFv antibodies recognizes H1N1 (A/Mallard/Italy/185/1996; A/Solomon Islands/3/2006) and H3N2 (A/Wisconsin/67/2005) strains, the clones AV.C4, AV.D1

Table 1
ELISA reactivity of the scFvs against H5N1 to different avian influenza viruses.

scFvs	Avian influenza viruses					
	H1N1 (A/Mallard/Italy/185/96)	H1N1[a] (A/Solomon Islands/3/06)	H3N2[a] (A/Wisconsin/67/05)	H5N2 (A/Mallard/Italy/80/93)	H5N3 (A/Mallard/Italy/	H5N1[b] (A/Vietnam/1194/04)
AV.G4	–	–	–	+	–	++++
AV.C4	–	–	–	+++	+++	++++
AV.A6	–	–	–	++	–	++++
AV.D1	–	–	–	++	+++	++++
AV.E2	–	–	–	+++	+++	++++

Microtiter plates were coated with the indicated antigens diluted in coating buffer and incubated ON at RT. After blocking, plates were incubated for 2 h at RT with supernatants containing soluble scFv antibody, anti-Flag M2 antibody and anti-mouse HRP conjugated antibody, washed, and later detected using POD-substrate (Roche Diagnostics). The reactivity was read with an ELISA reader at absorbance of 450 nm [+, above background and <2-fold; ++, between 2- and 3-fold; +++, between 3- and 10-fold; ++++, >10-fold above background; –, not measurable above background].

[a] Mutagrip Pasteur vaccine (season 2007–2008).

[b] Reverse genetically modified reassortant of H5N1 also named H5N1/NIBRG-14.

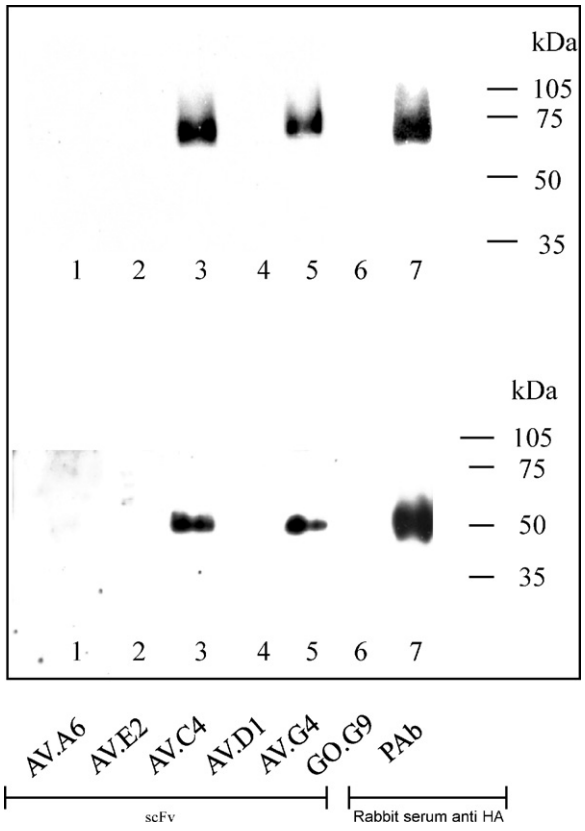

Fig. 3. Western blot of scFv antibodies. The anti H5N1 scFvs antibodies were analyzed by Western blot for H5N1 protein recognition in non-reducing (A) and reducing (B) conditions. Note that the scFv clones AV.C4 and AV.G4 bind with proteins with molecular mass of about 70 kDa (A) and 50 kDa (B) corresponding to the molecular weight of the H5 hemagglutinin protein and its major subunit HA1, respectively. Monospecific rabbit immunoserum raised against H5 polipeptide (lane 7) and scFv to glucose oxidase (lane 6) were used as positive and negative controls, respectively.

and AV.E2 bound with a conserved epitope shared by three different virus strains belonging to the H5 subtype, i.e. the reassortant H5N1/NIBRG-14 (A/Vietnam/1194/2004), and two viruses isolated from wild birds, A/Mallard/Italy/80/1993 (H5N2) and A/Mallard/Italy/208/2000 (H5N3). Conversely the scFv clones AV.G4 and AV.A6 displayed a more restricted recognition pattern since no reactivity with the H5N3 strain was observed. Western blot analysis showed that the scFv clones AV.C4 and AV.G4 detected protein bands of about 70 and 50 kDa in reducing and non-reducing condition, respectively. These biochemical patterns match well with the MW of the HA protein (Fig. 3A) and its major HA1 subunit (Fig. 3B). This hypothesis is supported by the similar biochemical pattern recognized by the polyclonal rabbit immunoserum against HA1 region of HA protein of H5N1 and used as a control (Fig. 3A and B).

3.3. Neutralization assay

The functional properties of the scFv antibodies were analyzed using the HI test against the H5N1/NIBRG-14 influenza virus, as previously described (Stephenson et al., 2003). Only the scFv clone AV.D1 demonstrated HI activity, even though at a very high concentration (data not shown). Since HI requires both antibody binding to, or nearby, the HA receptor binding site (RBS), and antibody ability to cause steric hindrance, the poor or absent HI activity observed in this study could be due to antibody binding outside the virus RBS as well as to a less effective barrier between virion and red blood cells posed by the reduced size of these immunoglobulin (Ig) fragments (27 kDa) compared to intact IgG (Schofield et al., 1997). It should be emphasized that even infection-neutralizing antibodies may have poor or absent HI activity (Imai et al., 1998; Kida et al., 1985; Okuno et al., 1993).

The H5N1 neutralization activity of the scFv antibodies was investigated in a pseudotype-based neutralization assay. Pseudotypes expressing HA from the homologous A/Vietnam/1194/2004 (clade 1), and from A/Indonesia/5/2005, A/whooping swan/Mongolia/244/2005 and A/Anhui/1/2005 H5N1 strains, representatives of subclades 2.1, 2.2 and 2.3 respectively, were incubated with serial dilution of purified scFv antibodies, and the reduction in luciferase activity in the target cells was measured in a single-round neutralization assay. Results are represented as the percentage of inhibition of infection of each pseudotyped-virus. The ID_{50} is calculated as the concentration of purified scFv antibodies giving the 50% reduction of infection. Under these conditions, the scFvs AV.D1 and AV.C4 were able to neutralize approximately 50% of A/Vietnam/1194/2004 pseudotype infection at the highest concentration tested, with an IC_{50} of 2.25 μg (Fig. 4A). Interestingly, the same scFv clones were able to fully neutralize the A/whooping swan/Mongolia/244/2005 and A/Indonesia5/2005 strains with an IC_{50} of 2.3 and 1.6 μg/ml, respectively (Fig. 4B and C), whereas no inhibition of infection was observed versus the clade 2.3 A/Anhui/1/2005 strain (Fig. 4D). All the other scFv antibodies (AV.A6, AV.E2 and AV.G4) did not interfere with the infection, and their neutralization activity was similar to that observed by using an irrelevant scFv antibody (in Fig. 4 the neutralization profile of scFv AV.A6 is representative of the negative clones).

4. Discussion

The prospect of passive immunotherapy for infectious disease has received considerable attention in recent years (Casadevall et al., 2004; Hanson et al., 2006; Sawyer, 2000). However, the administration of immunosera or monoclonal antibodies generated via conventional hybridoma technology, while feasible and effective, presents several limitations due to their xenogenic origin. Hence, these monoclonal antibodies require expensive and highly complex genetic manipulations to generate chimeric and/or humanized mAbs with lower but still unwanted immunogenicity.

All studies describing human neutralizing antibodies against influenza H5N1 have utilized whole immunoglobulins generated via EBV immortalization of human lymphocytes (Simmons et al., 2007) or genetic recombination of Fab fragments with the human IgG isotype (Hanson et al., 2006). Unfortunately EBV-immortalized cells are characterized by phenotypic instability, poor secretion, and pose important safety concerns for the presence of a potential infective agent and/or its degradation product in the drug substance (Center for Biologics Evaluation and Research, Food and Drug Administration, 1997; Committee for Medicinal Products for Human Use, European Medicines Agency, 2007). Recently, fully human monoclonal immunoglobulins have been obtained by genetically combining selected Fab fragments from naïve or immune phage antibody libraries (Lim et al., 2008; Kashyap et al., 2008). These engineered mAbs were transiently expressed in mammalian recipient cells and proved effective in in vitro/vivo H5N1 neutralization assay only as intact 150 kDa immunoglobulins. The development of these mAbs as a drug still presents critical biotechnological challenges, such as the isolation of a stable source to generate a reliable cell bank system.

The construction of libraries of recombinant antibody fragments that are displayed on the surface of filamentous phage has become an important technological tool in generating new monoclonal antibodies with clinical potential (Hoogenboom, 2005). By using this approach, rodent immunoglobulin fragments (Hanson et al., 2006) and human Fab (Lim et al., 2008; Kashyap et al., 2008) were iso-

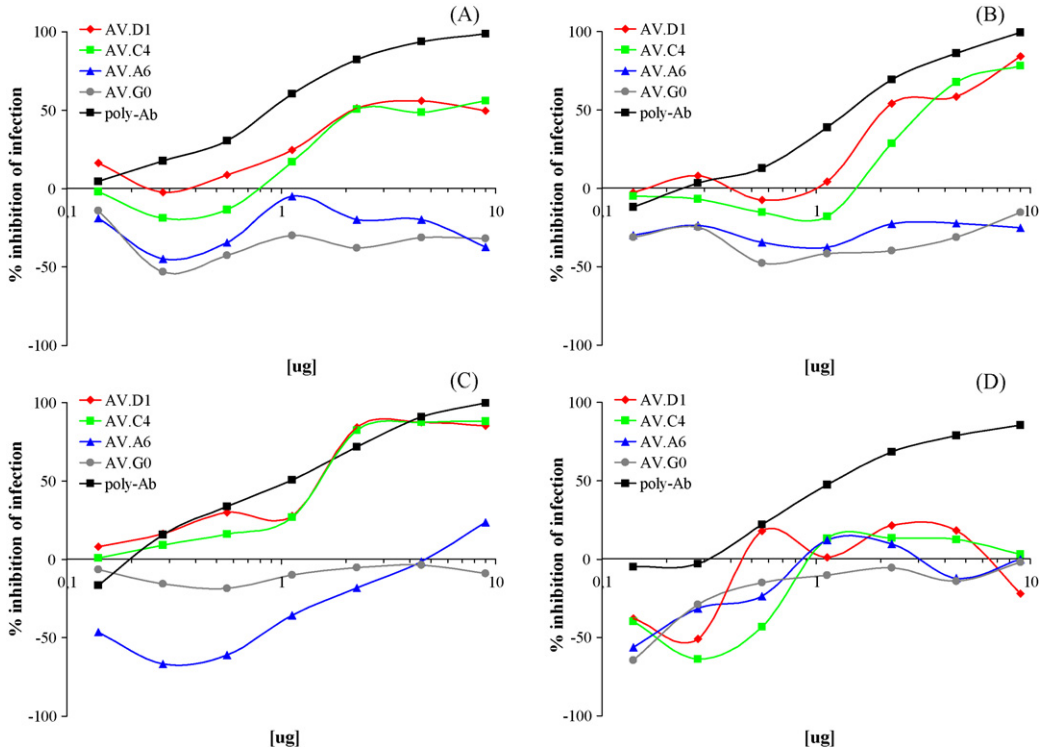

Fig. 4. Neutralizing activity of the scFvs against H5N1 pseudotypes. Pseudotypes expressing HA from A/Vietnam/1194/2004 (A), A/Mongolia/244/2005 (B), A/Indonesia/5/2005 (C) and A/Anhui1/2005 (D), were incubated with serial dilutions of the indicated scFv antibodies. The percentage of inhibition of infection was defined as the reduction in RLU compared to virus control wells after subtraction of background RLU. Sheep immune-serum against H5N1 by NISBC (poly-Ab) and irrelevant scFv GO against glucose oxidase were used as a positive and negative control, respectively. The profile of scFv AV.A6 is representative of the non-neutralizing clones.

lated and expressed as a portion of functional immunocompetent proteins against H5N1. Applying a similar approach and a semi-synthetic human antibody library constructed with the principles of protein design (Viti et al., 2000), we have shown for the first time that human scFv antibody fragments endowed with broadly neutralizing properties against H5N1 can be easily obtained from an entirely artificial antibody repertoire using reassortant BPL-inactivated H5N1/NIBRG-14 virus as a bait. Chemical procedures may significantly affect the structure of conformational epitopes and the immunogenicity of pathogenic virus. However, BPL-based procedures present the major advantage of allowing a safe handling of H5N1 viral proteic particles without affecting their antigenic potential as is demonstrated by effective vaccines in birds (Swayne et al., 2007) and the induction of a large array of site-specific B-cell immune responses in mice that generated protective mAbs via conventional hybridoma technology (Smirnov et al., 2004).

Pseudotyped viral particles are a widely used tool to express glycoproteins from a wide variety of 'hard to manage' viruses and are often used in neutralization assays as a safe surrogate of the virus (Li et al., 2005; Kong et al., 2006; Flint et al., 2004). For function analysis we generated pseudotype viruses bearing HAs from representative H5N1 viruses belonging to phylogenetic lineages (clade 1, clade 2.1, clade 2.2 and clade 2.3) responsible for human cases in different geographical areas (World Health Organization Global Influenza Program Surveillance Network, 2005, World Health Organization, 2007). By using this methodology two out five H5N1 positive scFv antibodies displayed a cross-clade neutralizing activity suggesting that the antibody immuno-chemical characteristics such as affinity and epitope recognition may be critical factors for function. In this regard, the genes encoding for scFv antibodies were isolated and sequenced, thus facilitating various molecular approaches, including site-directed mutagenesis to mature binding affinity or to construct multivalent recombinant antibodies in an effort to enhance or extend the neutralizing properties.

In conclusion, the scFv antibodies herein presented and discussed meet important criteria for a potential anti-H5N1 compound: they are human, hence poorly or not in the least immunogenic. In addition they neutralize the H5N1 virus in an in vitro "pseudotype-based" neutralization assay and can be produced on a large scale by using stable and safe prokaryotic cell systems. Furthermore, the small molecular size should provide for efficient tissue penetration, yet give rapid plasma clearance (Holliger and Hudson, 2005). Nonetheless, further studies will be addressed at better characterizing the capability of the scFv antibodies to neutralize live virus and to design an effective immunotherapy to combat H5N1 infection in humans.

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