VH (nanobody) directed against human glycophorin A: A tool for autologous red cell agglutination assays


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The preparation of a VH (nanobody) named IH4 that recognizes human glycophorin A (GPA) is described. IH4 was isolated by screening a library prepared from the lymphocytes of a dromedary immunized by human blood transfusion. Phage display and panning against GPA as the immobilized antigen allowed isolating this VH. IH4, representing 67% of the retrieved VH sequences, was expressed as a soluble correctly folded protein in SHuffle Escherichia coli cells, routinely yielding approximately 100 mg/L fermentation medium. Because IH4 recognizes GPA independently of the blood group antigens, it recognizes red cells of all humans with the possible exception of those with some extremely rare genetic background. The targeted linear epitope comprises the GPA Y52PPE55 sequence. Based on surface plasmon resonance results, the dissociation constant of the IH4–GPA equilibrium is 33 nM. IH4 is a stable protein with a transition melting temperature of 75.8°C (measured by differential scanning calorimetry). As proof of concept, we fused HIV p24 to IH4 and used the purified construct expressed in E. coli to show that IH4 was amenable to the preparation of autologous erythrocyte agglutination reagents: reconstituted blood prepared with serum from an HIV-positive patient was readily agglutinated by the addition of the bifunctional reagent.

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VHs, also known as nanobodies, are variable domains derived from heavy chain antibodies present in camels. Recombinant VHs are obtained by screening libraries prepared from the lymphocyte RNA of naive or immunized animals; they are renowned for their easy cloning and expression, and they have many applications in research, therapy, and diagnosis [1–3].

Aiming to obtain VHs directed against various antigens present on human red blood cells (RBCs),1 we immunized a dromedary by transfusion of human blood and derived a VH library from the animal’s lymphocytes. Because VHs that indiscriminately recognize RBCs of all humans may have interesting applications, we screened the library against glycophorin A (GPA), a protein present

1 Abbreviations used: RBC, red blood cell; GPA, glycophorin A; GPR, glycophorin B; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HA, hemagglutinin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS–T, PBS containing 0.05% Tween 20; PE, periplasmic extract; TBS, Tris-buffered saline; BSA, bovine serum albumin; PBS–T, PBS containing 0.05% Tween 20; IgG, immunoglobulin G; PBS–NaCl, PBS with 0.3 M NaCl; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; PBS–BSA, PBS containing 0.1% BSA; PVDF, polyvinylidene fluoride; SPR, surface plasmon resonance; DSC, differential scanning calorimetry; MFI, mean fluorescence intensity; scFv, single-chain variable fragment.
at a high copy number on RBCs and isolated several V\textsubscript{H}Hs; one of them that represents 67% of all isolated sequences was fully characterized and is described here.

GPA and glycophorin B (GPB) are almost exclusively expressed on RBCs, with as many as 800,000 copies/cell of GPA and 200,000 copies/cell of GPB \cite{4,5}. GPA and GPB are single transmembrane domain proteins with heavily glycosylated extracellular domains. GPA and GPB associate in the RBC membrane, hence, homodimers as well as heterodimers of GPA and GPB are present in the membrane, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of RBC membrane extracts. Glycophorins carry several blood group antigens, with the most important being M and N blood group antigens on GPA, and N, S, and s antigens on GPB. Moreover, glycophorins carry antigenic determinants that are independent of blood group antigens, and several murine monoclonal antibodies that target such constantly expressed epitopes were obtained \cite{6}.

We present data demonstrating that a V\textsubscript{H}H we have isolated may be used for autologous red cell agglutination assays \cite{7–9}. In short, monovalent V\textsubscript{H}H fused to an antigen, when added to whole blood taken from a patient, may induce red cell agglutination if the patient’s plasma contains antibodies to the fused antigen.

**Materials and methods**

**Immunization of dromedary**

RBCs from two human donors were pooled to present the most complete array of blood group antigens. The packed RBCs (300 ml) were transfused into a young female dromedary via jugular puncture. Transfusion was completed in approximately 1 h, with the animal experiencing only transient tremors. Next, 7 and 14 days later, 30 ml of the same pooled RBCs were injected subcutaneously without any clinical symptom.

**Preparation of antigens used for library panning**

Purified GPA used for panning was prepared essentially as described previously \cite{10}. In short, RBCs washed in 5 mM phosphate buffer (pH 8.0) were suspended in an equal volume of the same buffer supplemented with 0.6 M NaCl (final concentration). To this suspension, 9 volumes of chloroform/methanol mixture (2:1, v/v) was added. The suspension was shaken for 30 min at room temperature before being left in the cold room overnight. The aqueous phase containing a mixture of glycophorins (A, B, C, and D) was recovered and clarified by centrifugation for 30 min at 40,000g in a JA 20 Beckman rotor. The clarified supernatant was dialyzed against several changes of 5 mM ammonium carbonate (pH 8.3) and finally lyophilized. Highly purified GPA was obtained by reverse phase liquid chromatography performed in a C4 column using trifluoroacetic acid/acetonitrile/water mobile phases. Carboxymethylation of the crude glycophorin mixture prior to high-performance liquid chromatography (and hence of Cys50 of GPA present at the interface of the GPB–GPA dimer) results in disruption of the GPA–GPB heterodimers and enables obtaining homogeneous, GPB-free GPA \cite{10}.

**Preparation of library**

One week after the last subcutaneous human RBC injection, blood (100 ml) was drawn from the dromedary, lymphocytes were isolated, and the library was prepared according to established methods (see Refs. \cite{11,12} and references cited therein). The vector for library preparation was pHEN4; it is coding for a PelB signal sequence, the cloned V\textsubscript{H}H followed by the hemagglutinin (HA)-tag, and then downstream an Amber stop codon, the M13 bacteriophage protein PIII gene. Cells used for library preparation and subsequent pannings were TG1 cells allowing ribosome reading through the Amber stop codon to produce VH–PIII fusions.

**Screening of V\textsubscript{H}H library**

The human red cell immunized dromedary’s V\textsubscript{H}H library was screened using phage display. To capture the V\textsubscript{H}H-displaying phages, GPA (100 µl of a 100-µg/ml solution in phosphate-buffered saline [PBS]) was coated overnight at 4 °C in the capture well of a high-binding 96-well enzyme-linked immunosorbent assay (ELISA) plate (Costar, Corning, Cherges, France). A control well was left uncoated. The next morning, the capture and control wells were washed three times with PBS containing 0.05% Tween 20 (PBS–T) and blocked with 2% skimmed milk in PBS for 1 h at room temperature. Then, phages prepared from the library in the presence of helper phage M13K07 (New England Biolabs, Ipswich, MA, USA) were incubated for 1 h at room temperature in capture and control wells. Wells were washed five times with PBS–T, and phages were eluted with alkaline triethylamine solution. Phages eluted from the capture well were amplified after rescue with M13K07 helper phage; three consecutive rounds of panning were done. After each of the panning rounds, phages eluted from the capture well and from the control well were serially diluted and used to infect TG1 cells. The dilutions were then plated onto Petri dishes to evaluate the antigen-specific panning enrichment. Several clones derived from the positive well were grown in liquid medium (1 ml of Terrific Broth) and used to prepare periplasmic extract (PE) for ELISA to check for the expression of GPA-reactive V\textsubscript{H}H.

**ELISA, Pepscan analysis**

ELISA plates were coated overnight with antigen (10 µg/ml, 100 µl/well). For blocking and washing, 1% skimmed milk and PBS–T, respectively, were used. Each plate was probed with V\textsubscript{H}H samples (either PE or purified protein at 0.1–10 µg/ml incubated for 1 h at room temperature); a positive control was also loaded on each plate (an anti-DARC V\textsubscript{H}H \cite{13}, either purified or prepared as a PE, was incubated in a DARC-coated well) along with a negative control (PE prepared from uninfected TG1 or a purified unrelated V\textsubscript{H}H incubated in a GPA-coated well). Bound V\textsubscript{H}Hs were revealed with murine anti-HA antibody (HA.11 clone 16B12, Covance, Brussels, Belgium) and alkaline phosphatase-tagged anti-mouse antiserum (Sigma, l’Isle d’Abeau, France); an i-Mark Bio-Rad microplate reader (Marnes-la-Coquette, France) recorded the color developing from the phosphatase substrate (Sigma).

The binding of purified IH4 to immobilized peptides was studied using general methods similar to those of ELISA \cite{13,14}. Briefly, octapeptides covering the GPA sequence from A\textsubscript{40} to C\textsubscript{176} were synthesized on the tips of plastic pins. The pins were first blocked in Tris-buffered saline (TBS) containing 2% bovine serum albumin (BSA). The pins are arranged according to the format of a 96-well plate so that all incubations could be done using ELISA plates. After blocking, pins were incubated overnight with purified IH4 (50 ng/ml in TBS containing 0.05% Tween 20 [TBS–T]). IH4 binding was assessed with the anti-HA murine antibody and alkaline phosphatase-tagged anti-mouse antibody (Sigma, l’Isle d’Abeau, France); an i-Mark Bio-Rad microplate reader (Marnes-la-Coquette, France) recorded the color developing from the phosphatase substrate (Sigma).
VH subcloning, expression, and purification

Two vectors shown in Supplementary Fig. 1 (see Supplementary material) were used for subcloning with the same set of restriction enzymes for both vectors (PstI and EcoRII, Fermentas, Thermo Scientific, Illkirch, France). These restriction sites are located in frameworks 1 and 4, respectively, and are preceded and followed by sequences coding for 4 conserved N-terminal and 3 C-terminal VH residues, respectively. pHEN6cpM [13] codes for a PeI sequence, the VH, an HA tag, and finally a polyhistidine tail. The pET28-b vector obtained from Novagen (Merck, Darmstadt, Germany) was modified to allow easy subcloning of VHs with PstI and EcoRII as follows. First, the unique EcoRII restriction site present in the Lacl gene was disrupted by introducing a silent mutation (QuikChange Mutagenesis Kit, Stratagene, Agilent, Santa Clara, CA, USA). Then, starting from a pHEN6cpM plasmid harboring a VH, the fragment coding for VH, HA-tag, and stop codon was amplified, adequately digested, and ligated into NdeI- and XhoI-digested mutated pET28-b. The resulting plasmid encodes (from 5’ to 3’) a polyhistidine tail, a thrombin cleavage site, the VH, and finally an HA-tag; it is used for routine subcloning of other VHs using the PstI and EcoRII sites.

The pHEN6cpM vector drives VH expression in periplasm. Cells used for expression of pHEN6cpM-coded VH were BL21 (purchased from Stratagene). Expression in periplasmic space of BL21 cells was obtained by inoculating 330 ml of TB medium present in a 1-L shake flask with three side baffles. When bacterial culture optical density reached 0.8 to 1.2, VHIHI production was induced by adding isopropyl thiogalactoside at 1 mM final concentration, and fermentation was allowed to proceed at 37 °C for 18 h. PE was prepared by suspending the bacterial pellet in 60 ml of Tris buffer (diluted 1:4 with water) and incubating with 90 ml of Tes buffer (0.2 M Tris, 0.5 mM ethylenediaminetetraacetic acid [EDTA], and 0.5 M sucrose, pH 8.0), shaking in the cold for 1 h, and then diluting with 90 ml of Tris buffer (diluted 1:4 with water) and incubating for another 1 h. PE was recovered by centrifugation. SDS–PAGE verified VH in the supernatant, and the pellet was discarded.

The pET28-derived vector was used for intracytoplasmic VH expression in SHuffle C3029H cells (New England Biolabs). These cells are engineered to facilitate cytoplasmic expression of disulfide-bonded proteins. Expression in cytoplasm of SHuffle cells was obtained by inoculating 330 ml of LB medium in a 1-L shake flask with 3 side baffles and incubation at 30 °C. When optical density of fermentation medium reached 0.4 to 0.8, production was induced by adding isopropyl thiogalactoside at 0.1 mM final concentration, the temperature was lowered to 20 °C, and fermentation was allowed to proceed for 24 h. Pelleted cells resuspended in 60 ml of PBS containing 0.3 M NaCl (PBS–NaCl) were broken by three passages at 20,000 psi in an Emulsiflex homogenizer (Avestin, Ontario, Canada). Extract was clarified by centrifugation.

VHIHI was purified from PEs or cytoplasmic extracts using a cobalt-loaded Talon column (Clontech, Mountain View, CA, USA). Dimensions of the column suited to process the VHIHI amount produced by 1-L fermentation of SHuffle cells were 1.6 cm inner diameter and 10 cm height; the flow rate was 2 ml/min, and the equilibration buffer was PBS–NaCl. After sample loading, the column was rinsed first with PBS–NaCl and then with PBS–NaCl containing 10 mM imidazole, and VHIHI was eluted with PBS–NaCl containing 0.5 M imidazole. All buffers were supplemented with 1 mM phenylmethyl sulfonyl fluoride (PMSF). Purification from PE was performed similarly, but the column equilibration buffer and the sample, before loading, were supplemented with MgCl2 at 5 mM (final concentration). VHIHI eluted from the immobilized metal column was desalted in a PBS-equilibrated G25 column or polished by chromatography on a Superdex S70 column (GE Healthcare, Uppsala, Sweden) equilibrated in PBS.

Fusion of HIV p24 to IH4

Assaying antibodies directed against p24 is widely used to establish immunization against HIV [15]. A synthetic gene encoding the VHIHI C terminus (3’ to the EcoRII restriction site), then (in frame) HIV-1 gag protein residues 10 to 238 (GenBank: AAD28912.1), an HA-tag, and finally a stop codon was prepared by MWG Biotech (Ebersberg, Germany). Optimized codons for E. coli expression were used; the construct was provided by the manufacturer as an insert in a common plasmid. The construct was retrieved by polymerase chain reaction (PCR), and EcoRII and XhoI sites were added at the ampiclon’s 5’ and 3’ ends, respectively. After digestion, the ampiclon was ligated to the adequately digested and dephosphorylated pET28-derived plasmid containing IH4. Sequencing verified that no unwanted mutations had been introduced during the process. IH4–p24 fusion was expressed in SHuffle cells and purified from soluble cytoplasm extract, as described above for IH4.

Preparation of other E. coli-expressed constructs

Soluble p24 for competition experiments was obtained by EcoRII and PstI digestion of plasmid encoding the IH4–p24 fusion and ligation of a short synthetic oligonucleotide to replace the excised VHIHI sequence. We obtained a p24 derivative containing the N-terminal polyhistidine tag and thrombin site, the C-terminal HA-tag, and some residues derived from the IH4 frameworks 1 and 4 encoded by the plasmid used as starting material.

To study epitope location on GPA extracellular domain, the DNA sequence coding protein residues from Ser15 to His67 of the mature protein was amplified from a plasmid available in the laboratory [16] and ligated to an early version of T7 plasmid coding for Staphylococcus aureus nuclease [13]. From this vector, deletion mutants were obtained using adequate primers and a QuikChange Mutagenesis Kit (Stratagene). Five (5 consecutive amino acids) deletions encompassing GPA sequence from residues 40 to 64 were made (obtained sequences are shown on Supplementary Fig. 2). Constructs were expressed intracellularly as soluble proteins in BL21 cells. Bacterial lysates were loaded onto SDS–PAGE gels and blotted onto membranes that were probed with IH4 and anti-nuclease antiserum.

Flow cytometry experiments to characterize IH4

IH4 reactivity toward RBCs was studied using erythrocytes with the common different M and N phenotypes obtained from the Centre National de Reference des Groupes Sanguins (Paris, France). RBCs were washed twice in PBS and resuspended with either purified IH4 or, as control, anti-GPA+B murine monoclonal antibody (10 µg/ml) (clone E3, Sigma). The suspension was left at room temperature for 1 h. After incubation with the primary antibody, RBCs were washed twice in PBS and incubated for another 1 h in the presence of anti-HA monoclonal antibody (clone 16B9, an ascitic fluid purchased from Covance, diluted 1:16,000). A control of RBCs incubated with anti-HA alone was also prepared. Cells were washed again and then incubated in the dark at room temperature with anti-mouse IgG fluorescein isothiocyanate (FITC)-tagged Fab (5 µg/ml in PBS containing 0.1% BSA [PBS–BSA], Beckman Coulter, Villepinte, France). After a final wash step in PBS, cells were analyzed by digital high speed analytical flow cytometry. RBCs were identified based on forward and side scatter characteristics using logarithmic amplification. Highly diluted anti-HA antibody and detection with FITC-conjugated Fab (as indicated above) ensured only minimal RBC agglutination. The excitation wavelength was 488 nm, and the FITC signal was collected with a 515/45 bandpass filter. Data were acquired with BD FACS Diva software (version
6.1.2) and analyzed using FlowJo software (version 7.2.5, Treestar, Ashland, OR, USA).

In a second set of experiments, RBCs (suspended in PBS at 20% hematocrit) were treated with bovine trypsin (T1005, Sigma) at concentrations ranging from 75 μg/ml to 5 mg/ml for 20 min at 37 °C before being washed and analyzed by cytometry as described above.

Western blots

Western blots of purified proteins or of aliquots taken at the purification steps was performed in a Novex semidry apparatus (Life Technologies, Carlsbad, CA, USA) on nitrocellulose (Schleicher & Schuell, Dassel, Germany) or polyvinylidene fluoride (PVDF) membranes (Millipore, Agilent).

Antibodies used to reveal transferred proteins were (as needed) anti-HA (16 B9 clone), a rabbit anti-nuclease antiserum (prepared in-house), a murine anti-polyhistidine monoclonal (Novagen), a murine anti-p24 monoclonal (AB9071, Abcam, Cambridge, UK), a murine anti-glycophorin B+A (3B27 clone [6]), and appropriate secondary peroxidase-tagged antibodies. Chemiluminescence detection (ECL Reagent Kit, GE Healthcare) was used throughout.

Surface plasmon resonance

Surface plasmon resonance (SPR) analysis of VH-H interactions used a Biacore X100 apparatus (GE Healthcare). Purified GPA was immobilized on a CM5 chip in the Fc2 channel to a level of 400 resonance units using amine coupling chemistry, as recommended by the manufacturer. Reference channel Fc1 was simply activated and deactivated with ethanolamine. Six different analyte concentrations were injected onto the chip for 180 s, and dissociation was allowed to proceed for 600 s. Data were analyzed using BIA Evaluation software associated with the apparatus.

Differential scanning calorimetry

Experiments were performed in a Nano DSC (differential scanning calorimetry) apparatus (TA Instruments, New Castle, DE, USA). Protein solutions were prepared at 1 mg/ml concentration in PBS. Heat flow was recorded as a function of temperature, which was raised at 1 °C/min from 12 to 110 °C. Values measured with PBS alone were subtracted from those measured with proteins.

Agglutination experiments

Testing for agglutination on glass tile was performed as follows. First, 50 μl of reconstituted blood (prepared by mixing 1 volume of patient or control serum with 1 volume of O Rh-washed RBCs) was mixed on a glass tile with 50 μl of reagent in PBS–BSA containing either IH51 (2–100 μg/ml), IH51 (2.5 μg/ml) and purified p24 (50 μg/ml), or IH4 (2–100 μg/ml). Control consisting of PBS–BSA alone was also prepared. The drops were mixed and flattened to spread on an approximately 1.5-cm-diameter circle, the glass tile was gently rocked back and forth, and agglutination visually evaluated 2 min later and photographed.

Results and discussion

Construction of VH library, retrieval, and purification of IH4

The VHs amplified from lymphocyte RNA of the dromedary immunized with human RBCs were cloned into a library of 2.2 × 10^9 independent colonies, among which 80% contain a phagemid with a VH-H-sized insert. After phage display and panning on purified GPA, PE ELISA identified 242 VH_H clones, out of 352 analyzed, that specifically recognized the antigen. 208 bona fide VH_H sequences were retrieved from colony PCR of positive clones. The 30 different sequences that were obtained might be classified into three distinct families differing in the CDR3 region. The VH_H (referred to as IH4), which is the subject of this article, was found 140 times and so accounted for 67% of the positively identified sequences.

IH4 was expressed in the periplasmic space of BL21 cells or in the cytoplasm of SHuffle cells and was purified on an immobilized cobalt column. Yields of retrieved purified protein ranged from 1 to 10 mg/L fermentation medium in the case of periplasmic expression in BL21 cells, whereas 90 to 120 mg/L was obtained from expression in SHuffle cells. Proteins produced from either kind of cells were undistinguishable by functional studies described below. SDS–PAGE of aliquots of fractions collected from the immobilized metal affinity columns are shown in Supplementary Fig. 3.

Fig. 1. Flow cytometry analysis of IH4 interaction with RBCs. (A) RBCs do interact with IH4 irrespective of the M and N blood group antigens carried by GPA. The color code used for identification of the tracings is indicated beneath the panel. Negative control (RBCs were incubated only with anti-HA antibody and tagged anti-mouse IgG Fab) corresponds to the gray-filled histogram. (B, C) M+N+ RBCs were treated before incubation with VH_H or antibody with different concentrations of trypsin (indicated beneath the panels). Results of panel B were obtained with IH4; MFI does increase with increasing concentrations of trypsin. Results of panel C were obtained with a commercial anti-GPA+B murine antibody; MFI does decrease with increasing concentrations of trypsin. Negative controls correspond to the gray-filled histograms.
Characterization of recognized epitope and measurements of IH4 affinity for GPA

IH4 does recognize GPA on red cells independently of blood group antigens carried by the molecule. This was established by flow cytometry using red cells of defined phenotypes. Results are shown in Fig. 1. Reactivity is similar whatever blood group phenotype (M+N+, M−N+, or M+N−) (Fig. 1A).

Cytometry also gave an indication regarding epitope recognized by IH4; treatment of red cells with trypsin did increase mean fluorescence intensity (MFI) in a trypsin concentration-dependent manner (Fig. 1B), strongly suggesting that the recognized epitope is located C terminal to Arg39, which together with Arg31 is the preferential site for trypsin cleavage of GPA on RBCs [4]. Trypsin cleavage removes most of the glycan part of GPA and probably facilitates access of VH to its cognate epitope. By contrast, reactivity of a commercial anti-GPA-B monoclonal recognizing an epitope that is present on the 29 N-terminal residues common to both proteins was markedly diminished by trypsin treatment, but it was not totally abolished because GPB remains undigested after incubation with trypsin [4] (Fig. 1C).

β-Elimination of sugars on Western blot membranes by alkaline treatment [17], likewise, demonstrated that IH4 reactivity with GPA increased when sugar chains were removed from protein (see Supplementary Fig. 4).

Western blots displayed in Supplementary Fig. 2 show that E. coli-expressed recombinant extracellular GPA fragment fused to S. aureus nuclease is recognized by IH4. This suggests that the epitope recognized on GPA is essentially formed by the polypeptide chain. Moreover, studies of IH4 reactivity toward deletion mutants of the extracellular domain showed that only one of the deletion mutants was not recognized, narrowing down the identification of the epitope; clearly, only deletion of the T30SVVP54 sequence of GPA abolishes interaction of IH4. Finally, studies using peptides synthesized on plastic pins (Fig. 2) demonstrated that the linear epitope comprises the YPPE55 sequence. To our knowledge, continuous epitopes for VhHS have been described only twice before [13,18]. However, our current and former [13] results suggest that continuous epitopes should be systematically sought because they might provide new tags for identification and purification of recombinant proteins [18].

IH4 affinity for GPA was evaluated with SPR using purified GPA as the ligand (see Supplementary Fig. 5). Data can be easily fitted using 1:1 Langmuir fit. The $K_a$ is 33.72 nM (with $k_a$ and $k_d$ of 5.73 × 10^6 M^-1 s^-1 and 0.019 s^-1, respectively).

Characterization of bifunctional p24–IH4 derivative and of HIV-1 p24

To evaluate how IH4 might be used as a building block of a reagent for an autologous RBC agglutination assay, we assembled a bifunctional derivative by fusing HIV-1 p24 to the C terminus of IH4 (this construct is called IH51). The isolated p24 construct was also prepared as a control in agglutination experiments. Fermentation in SHuffle cells, purification of IH51, and p24 construct were performed like IH4. Yields of IH51 and of isolated p24 were both in the range of 100 to 120 mg/L fermentation medium. Lanes 2 and 3 in Fig. 3 show Coomassie-stained SDS–PAGE and Western blots of p24 and IH51, respectively.

As expected, purified proteins were recognized on Western blots by murine anti-p24 and the anti-HA-tag antibodies; moreover, human serum known to contain anti-p24 antibodies bound isolated p24 construct (lanes 2 in Fig. 3) and the bifunctional IH4–p24 fusion protein (lanes 3 in Fig. 3).

DSC of IH4 was repeated eight times, yielding identical results. The record shown in Fig. 4 demonstrates that IH4 is a very stable molecule with a transition temperature ($T_m$) of 75.8 °C (significantly higher than the highest $T_m$ of 72.5 °C that, to our knowledge, was published for a single-chain variable fragment [scFv] [19]). By contrast, a p24 $T_m$ of 39 °C indicates a somewhat labile protein; notably, a similar $T_m$ was published previously for p24 [20], suggesting that the presence of an N-terminus extension of our construct (the polyhistidine tag and a few VhH-derived residues) and the C-terminal HA-tag do not affect p24 thermostability. Finally, the DSC tracing of the bivalent fusion protein shows essentially a double peak with $T_m$ values of 42.6 and 58.5 °C, suggesting that fusion stabilized p24 domain and destabilized IH4 domain. It might be interesting to check whether the addition of a spacer between the two partners would influence thermostability of the
bifunctional molecule. More generally, our results suggest that the design of bifunctional molecules for auto-agglutination assays should take into account thermostability of each partner and of the resulting fusion protein. To increase the stability of the antigenic part, if necessary, it might be considered to use, when possible, peptides instead of full-size proteins; peptides might be epitope peptides derived from the parent protein or synthetic peptides identified as mimotopes of the antigen target (see Ref. [21] and references cited therein).

However, it is noteworthy that, even though IH4 present in IH51 seems to be less stable than unfused IH4, SPR experiments on IH4–p24 indicated a $K_D$ of 13.9 nM, close to the value measured for unfused IH4 (33.7 nM).

**Conclusions**

This article has described the first $\text{V}_{\text{H}}$ isolated after immunization of a camelid against human blood. The presented data focused on a $\text{V}_{\text{H}}$ that recognizes a linear GPA epitope unrelated to the blood group determinants borne by this protein. Therefore, this $\text{V}_{\text{H}}$ reacts with RBCs of all humans, with the possible exception of very rare individuals who do not express GPA in their RBCs or who express only a recombined version of GPA lacking the recognized sequence $Y_{52}\text{PPE}_{55}$ [23,24].

That the described $\text{V}_{\text{H}}$ was able to recognize RBCs of all humans suggests that it might be an excellent substitute for Fab- and scFv-derived reagents used as building blocks of reagents for autologous RBC agglutination assays [7–9]. $\text{V}_{\text{H}}$ would have distinct advantages because of the easy preparation and good yield of our constructs, unlike the multistep procedures needed to produce Fab- and scFv-derived reagents [7–9]. In this regard, it should be mentioned that SHuffle cells did allow us to produce several tens of milligrams per liter of fermentation medium of IH4–p24 fusion and of IH4 as soluble and functional proteins. Commercially available SHuffle cells were comprehensively described only recently [25]. They were developed on a classical BL21 background and engineered to diminish cytoplasmic reductive pathway and to express a

**Agglutination**

Reconstituted blood prepared from HIV-positive and control serum was used in agglutination experiments. A variety of techniques were tested, such as filtration on gel columns and agglutination in polystyrene plates [22], and all gave consistent results. Fig. 5 shows those obtained using the simplest and quickest glass tile technique. Agglutination is obvious in the drop containing 2.5 μg/ml IH51 fusion protein. A high concentration of reagent does inhibit agglutination; this fact is clearly attributable to competition between soluble IH51 and RBC-attached IH51 for binding of antibody present in reconstituted blood because the addition of free p24 construct to IH51 inhibits agglutination (see left drop of middle row). Agglutination was dependent on the presence of specific antibody in the patient’s plasma because no agglutination whatsoever was observed with control plasma from a healthy donor (lower row); agglutination depends on the presence of p24 antigen fused to IH4 because IH4 alone at any concentration does not induce agglutination (only one concentration is shown in the middle row).
protein disulfide isomerase. Other previously published work [26] showed that coexpression of both a protein disulfide isomerase and a sulphydryl oxidase might also allow high yield production of V₃H. In our hands, every V₃H produced to date in SHuffle cells was expressed in high yield and mostly in soluble form [27]. We also produced Plasmodium falciparum proteins that are notoriously difficult to express because of their numerous disulfide bonds [28].

Using IH4 as the fusion partner, it was possible to design a reagent applicable in autologous RBC agglutination assays. Indeed, the presence of antibodies to p24 could be demonstrated within minutes using a very simple procedure. This experiment on one single-plasma sample and detecting reactivity to a single antigen [15] is a proof of concept. We did not study a large number of samples or compare results with more established techniques such as ELISA because comparisons of performances of Fab- and scFv-based autologous RBC agglutination tests were published previously [9,29]. Although the principle underlying autologous RBC agglutination tests was described many years ago and its applicability to AIDS diagnosis was demonstrated [7–9], it is not currently established as a reference technique and no agglutination assay is recommended for diagnosis of AIDS by the Centers for Disease Control and Prevention [30]. The reason for the relative lack of recognition is not obvious, the relative complexity of preparation of autologous single-plasma sample and detecting reactivity to a single antigen [15] is a proof of concept. We did not study a large number of samples or compare results with more established techniques such as ELISA because comparisons of performances of Fab- and scFv-based autologous RBC agglutination tests were published previously [9,29]. Although the principle underlying autologous RBC agglutination tests was described many years ago and its applicability to AIDS diagnosis was demonstrated [7–9], it is not currently established as a reference technique and no agglutination assay is recommended for diagnosis of AIDS by the Centers for Disease Control and Prevention [30]. The reason for the relative lack of recognition is not obvious, the relative complexity of preparation of autologous single-plasma sample and detecting reactivity to a single antigen [15] is a proof of concept. We did not study a large number of samples or compare results with more established techniques such as ELISA because comparisons of performances of Fab- and scFv-based autologous RBC agglutination tests were published previously [9,29].

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jab.2013.03.020.

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