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Monovalent single-chain Fv fragments and bivalent miniantibodies bound to vesicular stomatitis virus protect against lethal infection

Several antibody-dependent mechanisms have been postulated to mediate neutralization of different animal viruses, including blocking of docking to receptors, induction of conformational changes in the virus coat, and Fc-dependent opsonization. We have studied the molecular requirements for antibody-mediated neutralization of vesicular stomatitis virus (VSV) *in vitro* and protection against lethal disease *in vivo* with a single-chain Fv fragment (scFv) and the corresponding bivalent miniantibody (scFv-dHLX) generated from a VSV-neutralizing monoclonal antibody. Both monovalent scFv and bivalent scFv-dHLX miniantibodies were able to neutralize VSV *in vitro* and to protect interferon-αβ receptor-deficient (IFN-$\alpha\beta R^{-/-}$) mice against lethal disease after intravenous injection of 50 plaque-forming units (pfu) VSV pre-incubated with the scFv reagents. Similarly, severe-combined immunodeficient (SCID) mice infected with immune complexes of 10^8 pfu VSV and bivalent scFv-dHLX were protected against lethal disease; however, mice infected with immune complexes of 10^8 pfu VSV and monovalent scFv were not. Although repeated scFv-dHLX treatment reduced virus quantities in the blood, neither SCID nor IFN-$\alpha\beta R^{-/-}$ mice were protected against lethal disease after passive immunization and subsequent VSV infection. This was due to the short half-life of 17 min of scFv-dHLX in the circulation. These data demonstrate that neutralization of VSV and protection against lethal disease do not require Fc-mediated mechanisms and that cross-linking is not crucial for protection against physiologically relevant virus doses *in vivo*.

1 Introduction

Vesicular stomatitis virus (VSV) belongs to the family of Rhabdoviridae and is closely related to rabies virus. VSV can infect a broad host range [1]. In immunocompetent mice, VSV may cause a progressive paralytic disease leading to death within 6–10 days [2, 3]. Protection against lethal disease is mediated by virus-neutralizing antibodies. After experimental infection of immunocompetent mice, high VSV-specific IgM titers are measurable by day 4 [4] and a strictly Th-dependent switch to IgG occurs between days 6 and 8 [5]. IgG titers produced during a primary infection stay elevated for life. Early VSV-specific antibodies bind to the viral glycoprotein with high affinity and neutralize VSV [6]. Neutralization of VSV is strictly serotype specific, as demonstrated for the serotypes Indiana (VSV-IND) and New Jersey (VSV-NJ) [1]. VSV-neutralizing antibodies are necessary and sufficient for protection against lethal infection of immunodeficient mice [7].

Previous experiments have shown that $F(ab')_2$ fragments generated proteolytically from VSV-specific antibodies neutralized VSV *in vitro* and were able to protect mice upon lethal virus challenge [3]. Recently developed recombinant antibody technology allows further reduction of the antibody fragment size to the minimal specificity-conferring Fv portion [8, 9]. Furthermore, recombinant antibody-fragment preparations have the advantage that contamination with whole antibodies can be ruled out.

Earlier studies have documented the activities of recombinant virus-specific Fab [10–12] and single-chain Fv fragments (scFv) *in vitro* [13, 14]. Several authors speculated that scFv reagents might have interesting features for therapeutic applications due to the reduced size, *e.g.* improved tissue penetration [15, 16]. The protective properties of recombinant virus-specific Fab fragments were evaluated *in vivo* for respiratory syncytial virus (RSV) and HIV-1 infections [17, 18]. However, only limited data are available about the activities of virus-specific scFv fragments *in vivo* [14].

To investigate VSV-specific binding and neutralization of scFv and to analyze the protective capacity of scFv reagents in a mouse model, we generated a monovalent scFv fragment and a bivalent miniantibody from the VSV-neutralizing mAb VI24 [6]. The properties of both constructs were analyzed *in vitro* and *in vivo*. Our studies revealed that neutralization of VSV *in vitro* and protection against lethal disease *in vivo* can be achieved indepen-

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Abbreviations: **VSV:** Vesicular stomatitis virus **VH:** Variable region of the heavy chain **VL:** Variable region of the light chain **scFv:** Single-chain Fv antibody fragment **scFv-dHLX:** Bivalent scFv miniantibody **IFN-$\alpha\beta R^{-/-}$:** Interferon-αβ receptor-deficient **SCID:** Severe combined immunodeficient $t_{1/2}$: Half-life

Key words: Vesicular stomatitis virus / Virus neutralization / Single-chain Fv

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dently of Fc-mediated functions and seem to depend upon covering of the virus surface.

2 Materials and methods

2.1 Cloning and expression of monovalent scFv and bivalent miniantibodies with VH and VL of the VSV neutralizing mAb VI24

The VSV-neutralizing mAb VI24 [6] was chosen to construct recombinant scFv fragments because the structure/function relationships of the VH and VL genes of this antibody have been studied in detail (U. Kalinke and A. Oxenius, unpublished). For cloning of the VL and VH genes of VI24, mRNA was isolated from 10^6 logarithmically grown hybridoma cells with a QuickPrep™ Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden). Poly(A^+) mRNA (1 μg) was transcribed to cDNA by random hexanucleotide-primed avian myeloblastosis virus reverse transcriptase (Amersham, Little Chalfont, GB). The cDNA was used as a template for PCR amplification of VL and VH. Both fragments were joined by PCR assembly and the 800-bp PCR fragment so obtained was ligated via compatible Sfi I sites into the phage display vector pAK100 [19]. The nucleotide sequence of the PCR-derived DNA (EMBL Nucleotide Sequence Database accession number X97498) in the resulting plasmid pLi20 was verified by automatic sequencing (Applied Biosystems 373, Foster City, CA). Functionality of the scFv was first analyzed by phage display. For this, *Escherichia coli* XL1-Blue (Stratagene, San Diego, CA) transformed with pLi20 was grown to $OD_{550} = 0.5$, infected with the helper phage VCS (Stratagene) and incubated at 37 °C overnight. VSV-specific binding of the culture supernatant containing recombinant scFv-displaying phages was demonstrated in a standard ELISA [6, 19] using sheep anti-M13 horseradish peroxidase-conjugated antibodies (Pharmacia) for detection of phages bound to VSV.

For bacterial expression of the histidine-tagged soluble scFv fragment of VI24 (scFv24) the 800-bp Sfi I fragment was cloned into the bacterial expression vector pAK300 [19], giving rise to the construct pscFv24. To obtain the construct pscFv24-dHLX for expression of the bivalent miniantibody, the Eco RI/Hind III fragment of pLi20 encoding the C-terminal portion of the g3p phage protein was replaced by a 182-bp Eco RI/Hind III fragment encoding a hinge peptide followed by a helix-turn-helix motif (dHLX) and a C-terminal his-tail [20] (Fig. 1A).

2.2 Expression and purification of recombinant scFv and miniantibodies

Two × YT medium supplemented with 1 % glucose and 30 μg/ml chloramphenicol was inoculated with an overnight culture of *Escherichia coli* JM83 (*ara, Δ(lac-proAB), strA, thi-1* [*Ø80lacZΔM15*]) harboring pscFv24 or pscFv24-dHLX, respectively. Cultures were grown with vigorous shaking at 25 °C. At a density of $OD_{550} = 0.5$, expression of scFv proteins was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and 3–4 h later, cells were harvested by centrifugation. The pellets were resuspended in 40 mM Hepes, 300 mM NaCl, 2 mM $MgCl_2$ pH 7.5 with 0.25 mg/ml RNase (Boehringer, Mannheim, Germany), 0.25 mg/ml DNaseI (Boehringer), 1.6 mg/ml hen egg lysozyme (Biomol, Hamburg, Germany). Then bacterial cells were disrupted with a French press and the lysate was cleared by centrifugation (20 000 rpm for 1 h at 4 °C) and filtration (0.22-μm pore filter; Millipore, Bedford, MA). The recombinant proteins, which contain an N-terminal FLAG-tag [21] and a C-terminal his-tag, were purified from the cleared lysates in two steps. In the first step the lysate was applied to a Ni-nitrilotriacetic acid Superflow column (Qiagen, Chatsworth, CA). The column was extensively washed with a buffer (20 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 0.2% Tween, pH 7.5) containing 50 mM imidazole and scFv24 and scFv24-dHLX were batch-eluted with 200 mM imidazole. In the second step, all fractions containing scFv24 or scFv24-dHLX of 50–60 % purity were directly applied to a FLAG column (Eastman Kodak, Rochester, NY). The column was washed with 20 mM Hepes containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5, and scFv24 and scFv24-dHLX were eluted with 100 mM glycine-HCl pH 3. The pH of the fractions was immediately neutralized by the addition of 1 M Tris base.

2.3 Mice and viruses

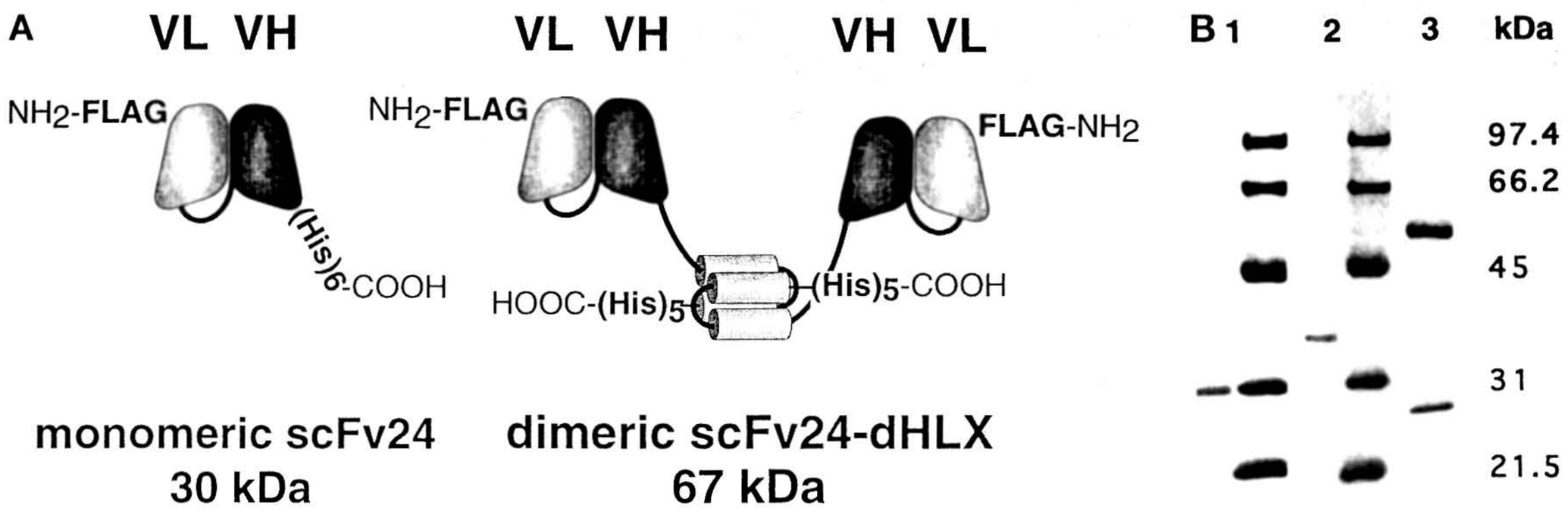
BALB/c-SCID mice 6–8 weeks old kept under specific pathogen-free (SPF) conditions were obtained from GSF (Oberschleissheim, Germany). Interferon-αβ receptor-deficient (IFN-$\alpha\beta R^{-/-}$) mice [22] were bred under SPF conditions at the Institut für Labortierkunde, University of Zürich, Switzerland. VSV-IND (Mudd-Summers isolate) was originally obtained from D. Kolakofsky, University of Geneva, Switzerland, and was grown on BHK-21 cells [23].

2.4 VSV neutralization test

Serial twofold dilutions of antibody samples were mixed with equal volumes of virus containing 500 pfu/ml. The mixtures were incubated for 90 min at 37 °C in air containing 5 % CO_2. Then, 100 μl of the mixtures were transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37 °C. The monolayers were overlaid with 100 μl Dulbecco's modified Eagle's medium containing 1 % methylcellulose. After incubation for 24 h at 37 °C, the overlay was removed and the monolayer was fixed and stained with 0.5 % crystal violet dissolved in 5 % formaldehyde, 50 % ethanol, and 4.25 % NaCl. The dilution reducing the number of plaques by 50 % was recorded as the neutralizing Ab titer [4].

2.5 Determination of virus titers from serum and brain tissue of infected mice

Serum was prediluted tenfold and further diluted in twofold steps. Mouse brains were homogenized in balanced salt solution (BSS) with a lab homogenizer and serial threefold dilutions were prepared. Then 100 μl of the serum or the homogenized brain tissue dilutions were transferred onto Vero cell monolayers in 96-well plates and the plates were further treated as described in Sect. 2.4. Virus titers were calculated by multiplying the number of plaques by the dilution factor.



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FLAG                                VL-CDR1                                VL-CDR2      70
DYKDIVMSQS PSSLAVSVGE KVTMSCKSSQ SLLYSSNQKN YLAWYQQKPG QSPKLLIYWA STRESGVPDR

                                VL-CDR3                  20 amino acid linker 140
FTGSGSGTDF TLTISSVKAE DLAVYYCQQY YSYYTFGGGT KLEIKRGGGG SGGGGSGGGG SGGGGSDVQL

                                VH-CDR1                VH-CDR2                  210
VESGGGLVQP GGSRKLSCAA SGFTFSNFGM YWVRQAPEKG LEWVAYISSG SSTIFYADTV KGRFTISRDN

                                VH-CDR3                His-tail
PKNTLFLQMT SLRSEDTAMY YCARSGGYSF DYWGQGTTLT VSSASGA - DHHHHHH


                                dHLX-motif                          His-tail
- EFP KPSTPPGSSG ELEELLKHLK ELLKGPRKGE LEELLKHLKE LLKGGSGGAP HHHHH
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Figure 1. Structural properties of monovalent scFv24 and bivalent scFv24-dHLX miniantibody. (A) Schematic depiction of monomeric scFv24 and bivalent scFv24-dHLX miniantibody. (B) SDS-PAGE analysis of purified scFv24 and scFv24-dHLX. Aliquots of 20 µl of purified scFv24 (lane 1), 15 µl scFv24-dHLX (lane 2) and 10 µl mAb VI24 (lane 3) were separated by SDS-PAGE under reducing conditions and the gel was stained with Coomassie blue. Monomeric scFv24 and bivalent scFv24-dHLX miniantibody were purified from bacterial lysates in two steps by immobilized metal ion chromatography and immunoaffinity chromatography. Whole mAb VI24 was purified from hybridoma supernatant by protein G-affinity chromatography. Between lanes 1, 2, and 3, protein standards with the indicated molecular masses were run. (C) Primary amino acid sequence of scFv24 and scFv24-dHLX miniantibody deduced from nucleotide sequences. They differ by the sequence after the dash (his-tail or dHLX motif with His-tail).

3 Results and discussion

3.1 Construction of monovalent scFv24 and bivalent scFv24-dHLX miniantibody

To generate a monovalent scFv fragment of mAb VI24, the VL and VH genes were cloned from cDNA and joined by a sequence encoding a flexible $(Gly_4Ser)_4$-linker by PCR amplification. The resulting PCR fragment was ligated into a bacterial expression vector. Bivalent scFv24 miniantibodies were obtained by introducing a hinge region followed by two tandemly repeated amphiphilic helices (dHLX motif) at the 3′ end of the scFv24 gene [20]. Dimer formation of two scFv24-dHLX molecules in *E. coli* is conferred by association of the dHLX motif to a four-helix bundle [20]. Both fusion proteins, scFv24 and scFv24-dHLX, were equipped with a short FLAG-tag (Asp-Tyr-Lys-Asp) at the N terminus [21] and a his-tail at the C terminus. A schematic depiction of monovalent scFv24 and bivalent scFv24-dHLX miniantibody is shown in Fig. 1A.

The recombinant scFv fusion proteins were purified from bacterial lysates in two steps, involving the binding of the his-tail to a metal-chelate column and binding of the FLAG-tag to an anti-FLAG affinity column [21]. As shown by SDS-PAGE analysis under reducing conditions, purified scFv24 migrated at 30 kDa (Fig. 1B, lane 1), scFv24-dHLX homodimers dissociated and mi grated at about 33 kDa (Fig. 1B, lane 2), and the whole antibody VI24 gave rise to two bands at 25 kDa for the light chain and at 50 kDa for the heavy chain (Fig. 1B, lane 3). Size exclusion chromatography experiments with purified scFv24 gave rise to one single protein peak with a relative retention V_e/V_o of 1.83, confirming the monomeric structure of scFv24 (Table 1). Due to hydrophobic properties of scFv24 and scFv24-dHLX (Fig. 1C), both proteins had a solubility limit of approximately 25 µg/ml, thus giving an upper limit of usable concentrations.

3.2 Neutralization of VSV-IND *in vitro* by monovalent scFv24 and bivalent scFv24-dHLX miniantibody

Both monovalent scFv24 and bivalent miniantibody neutralized VSV-IND in a VSV neutralization test *in vitro* (Table 2). The fourfold better neutralization of the bivalent miniantibody at the same molar concentration of binding domains suggested some, albeit limited, contribution to binding strength by multivalency. This observation is in agreement with an earlier comparison of VSV-specific binding of the mAb VI24 and the Fab fragment, which differed only by a factor of 2 [6].

The comparison of the same molar concentration of binding domains indicated that whole mAb VI24 neutralized

Table 1. Size exclusion chromatography of monovalent scFv24[a)]

	Molecular mass [kDa]	Relative retention [V_e/V_o]
scFv24	30.0	1.83
Lysozyme	14.4	2.40
Trypsin inhibitor	21.5	2.08
Carbonic anhydrase	31.0	1.69
Ovalbumin	45.0	1.61

a) Purified scFv24 (20 µl) and a protein standard (Bio-Rad) were applied to a Superose 12 column (Pharmacia) equilibrated with BBS (200 mM H_3BO_4, 120 mM NaCl, pH 8.0) and analyzed in a Pharmacia Smart System. The elution volume V_e was divided by the void volume V_o (0.85 ml) which was determined with Blue Dextran (Pharmacia). The relative retention of scFv24 indicated a homogenous preparation of a monomeric protein of approximately 30 kDa. The calculated molecular mass of scFv24 is 28.2 kDa (Fig. 1C).

Table 2. VSV-IND neutralization titer of whole mAb VI24, scFv24, and scFv24-dHLX miniantibody

Antibody	Neutralization titer[a)]
VI24	13
scFv24 (monovalent)	9
scFv24-dHLX (bivalent)	11

a) Log_2 dilution of 25 µg/ml purified protein neutralizing 50 % of input virus.

VSV approximately tenfold better than the single-chain miniantibody (Table 2). This difference is probably due to improperly folded or denatured protein in the scFv24-dHLX preparation, which is not available for functional binding. Nevertheless, functional molecules in the scFv24-dHLX preparation displayed the expected fine specificity of the parental mAb VI24. This was verified by inhibition of the VSV-IND-specific binding of the mAb VI24 by scFv24-dHLX in a binding competition assay (data not shown). Thus, the data demonstrate that VSV neutralization *in vitro* is independent of Fc-mediated functions, and it does not require bivalent binding. A high degree of covering of the virus surface seems to be sufficient for neutralization of VSV *in vitro*.

3.3 Analysis of protection against lethal VSV infection by monovalent scFv24 and bivalent scFv24-dHLX miniantibody

To analyze whether scFv24 or bivalent miniantibody bound to VSV are able to protect against lethal disease, 10^8 pfu VSV-IND were incubated with 2 µg mAb VI24 or 5 µg scFv24 or 5 µg scFv24-dHLX for 30 min at room temperature in a total volume of 300 µl before i.v. transfer to immunodeficient SCID mice. Immunodeficient mice were used to avoid any complications from an induced IgM response which would occur during the first 4 days. After 4 days, mice were killed and virus titers were determined in the brain.

SCID mice treated with immune complexes from VSV and mAb VI24 did not show any virus in the brain after 4 days, whereas SCID mice which were injected with VSV alone showed high virus titers of about 10^8 pfu in the brain (Fig. 2A). After injection of VSV pre-incubated with monovalent scFv24, SCID mice showed a delayed progression of disease, and after 4 days exhibited 10^5 pfu virus in the brain which was reduced 100-fold compared to controls (Fig. 2A). However, these mice eventually died, *i.e.* they were not protected against lethal infection. SCID mice injected with VSV and bivalent scFv24-dHLX immune complexes did not show any virus in the brain after 4 days (Fig. 2A). Thus, SCID mice were protected against lethal disease after exposure to 10^8 pfu VSV pre-incubated with 5 µg bivalent scFv24-dHLX or 2 µg mAb VI24, but not after treatment with VSV pre-incubated with 5 µg monovalent scFv24.

We then analyzed whether bivalency added a new quality to the VSV-specific binding or just improved avidity above the critical threshold in this experiment. Such a new binding quality might involve conformational changes by cross-linking of glycoproteins on the virus surface or agglutination of several virus particles, thereby dramatically reducing the overall number of pfu. To test this possibility, a second model situation was analyzed which required minimal virus doses for a lethal infection. IFN-$\alpha\beta R^{-/-}$ mice, which die after infection with as little as 5 pfu VSV-IND within 3–4 days [24], were treated i.v. with 50 pfu VSV-IND pre-incubated with 1.25 µg scFv24 or 1.25 µg scFv24-dHLX for

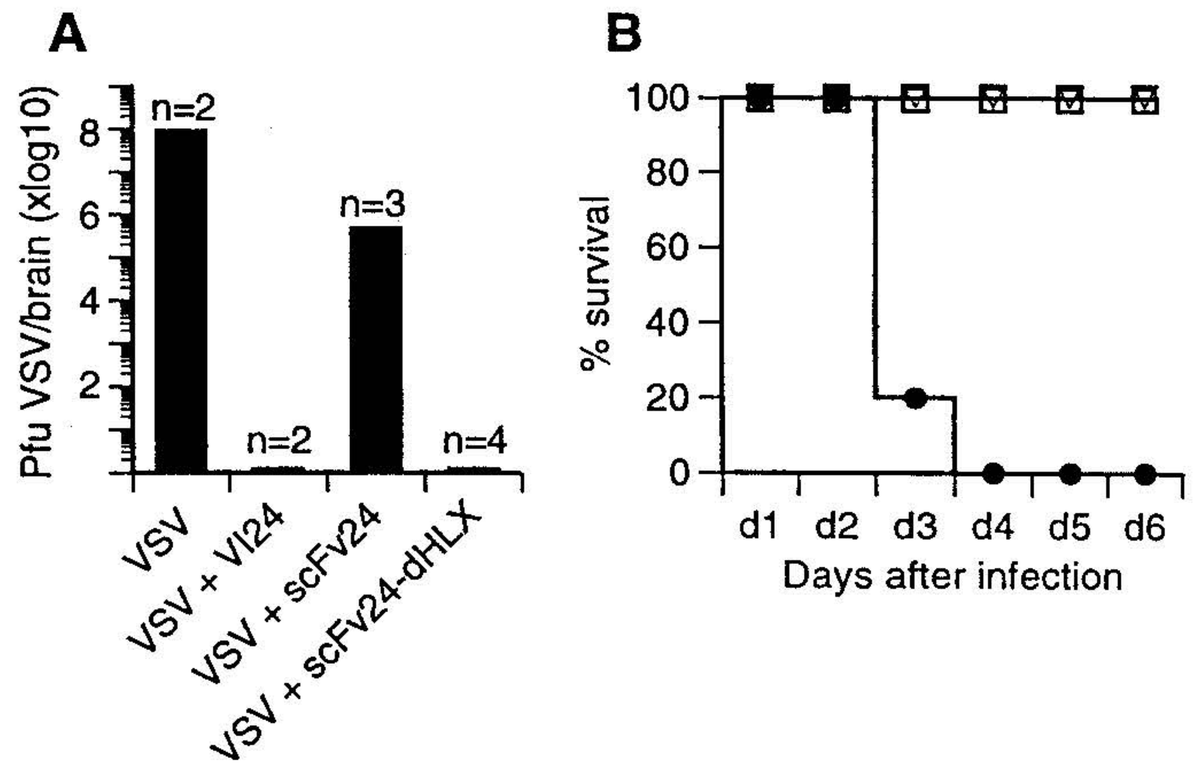

Figure 2. Analysis of protection against lethal disease after injection of preformed immune complexes of VSV-IND and monovalent scFv24 or bivalent scFv24-dHLX miniantibody. (A) Virus titers in brains of SCID mice 4 days after i.v. inoculation of 10^8 pfu VSV-IND pre-incubated for 30 min at room temperature with 2 µg mAb VI24 (VSV + VI24), with 5 µg monovalent scFv24 (VSV + scFv24), or with 5 µg bivalent scFv24-dHLX (VSV + scFv24-dHLX) in a total volume of 300 µl. A control group of two SCID mice received 10^8 pfu VSV-IND i.v. after incubation for 30 min at room temperature (VSV). The size of the different groups is indicated. One of two similar experiments is shown. (B) Percent survival after indicated time: groups of five IFN-$\alpha\beta R^{-/-}$ mice which were either infected with 50 pfu VSV (filled circles) or treated i.v. with 50 pfu VSV-IND pre-incubated for 30 min at room temperature with 1.25 µg monovalent scFv24 (open squares) or with 1.25 µg bivalent scFv24-dHLX miniantibody (open triangles) in a total volume of 100 µl. One of two similar experiments is shown.

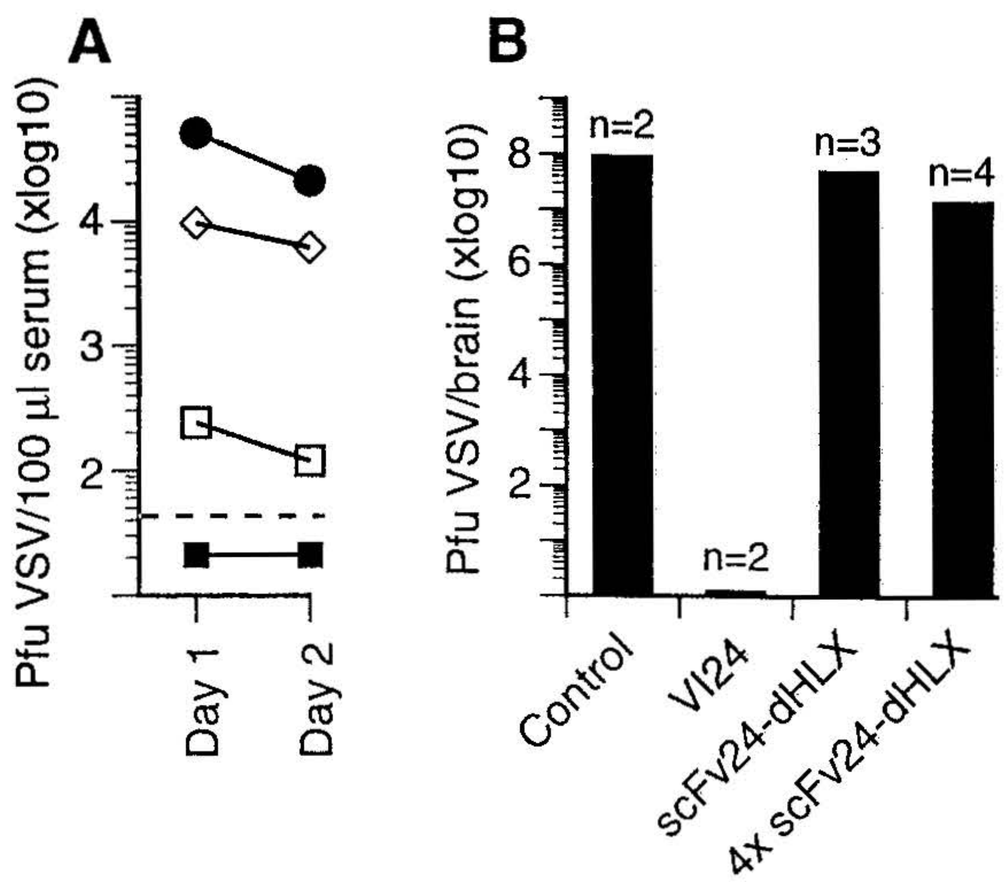

Figure 3. Virus titer in SCID mice after passive immunization and subsequent VSV infection. (A) Virus titer in blood of SCID mice 1 and 2 days after infection. SCID mice were passively immunized in the left tail vein with 2 µg mAb VI24 (VI24, filled squares) or 5 µg scFv24-dHLX (scFv24-dHLX, open diamonds); 15 min later, treated mice and untreated control animals (Control, filled circles) were infected with 10^8 pfu VSV-IND in the right tail vein. Another group of mice was treated four times with 5 µg scFv24-dHLX each every 2 h beginning with the first treatment 15 min before infection with VSV (4 × scFv24-dHLX, open squares). Virus titers were determined in a standard plaque assay. The detection limit of the assay is indicated by the dotted line. One of four similar experiments is shown. (B) Virus titer in the brain of the same SCID mice from (A) 4 days after infection. n = number of mice per group. One of two similar experiments is shown.

30 min at room temperature in a total volume of 100 µl. As expected, control mice infected with 50 pfu VSV-IND died between days 3 and 4 (Fig. 2B). In contrast, mice treated with 50 pfu VSV-IND pre-incubated either with monovalent scFv24 or bivalent scFv24-dHLX miniantibody did not show any signs of disease and survived more than 6 days (Fig. 2B). Mice killed on day 6 contained no virus in the brain or other tissues (data not shown). Comparable data were obtained with infection doses of 500 pfu VSV-IND (data not shown). Thus, both monovalent scFv24 and bivalent scFv24-dHLX miniantibody bound to VSV were able to protect IFN-$\alpha\beta R^{-/-}$ mice from lethal disease, indicating that bivalency is not an absolute requirement for protection *in vivo*. These findings are in contrast to experiments with Sindbis virus, which showed that bivalency is crucial for clearance of infectious virus from neuronal cells [25].

Next, passive immunization with the bivalent scFv24-dHLX miniantibody and the bivalent parental mAb VI24 was examined. SCID mice were treated with 2 µg VI24 or 5 µg scFv24-dHLX, and 15 min later, they were infected via the contralateral vein with 10^8 pfu VSV-IND. VSV was determined in blood 1 and 2 days after infection. On day 4, mice were killed and the VSV content of the brain was measured. SCID mice infected with VSV without prior antibody treatment showed VSV in blood (Fig. 3A) and high titers in the brain (Fig. 3B), and died between day 2 and 4. In contrast, SCID mice which were passively immunized with mAb VI24 and then infected with VSV did not contain any virus, either in the blood or in the brain, after 4 days. SCID mice treated once with scFv24-dHLX before VSV infection showed virus titers in the serum which were slightly reduced compared to untreated mice (Fig. 3A), but by day 4, virus titers in the brain were as high as in antibody-free controls (Fig. 3B). Despite substantially reduced virus titers in the serum of SCID mice after four scFv24-dHLX treatments repeated at intervals of 2 h, starting with the first treatment 15 min before VSV infection, the brain showed high virus titers on day 4. Thus, repeated passive immunization with bivalent scFv24-dHLX miniantibodies reduced and delayed virus replication significantly by a factor of 100 during the first 2 days. However, repeated passive immunization did not suffice to prevent virus from entering neuronal tissues, and therefore did not protect against lethal disease. Comparable results were obtained with IFN-$\alpha\beta R^{-/-}$ mice (data not shown).

To elucidate why scFv24-dHLX pre-bound to VSV protected against disease and scFv24-dHLX given as a passive immunization before VSV infection did not, the half-life of VI24 and scFv24-dHLX in the circulation of SCID mice was measured. SCID mice were treated i.v. with 2 µg mAb VI24 and 10 µg scFv24-dHLX. Blood samples were taken at indicated time points and serum was tested for VSV-neutralizing activity. During the observation period of 4 days, the neutralizing titer measured after immunization of VI24 did not change significantly. In contrast, in mice injected with scFv24-dHLX miniantibodies, the neutralization titer disappeared from the circulation with a half-life of about 17 min (Fig. 4). Dissociation of scFv24-dHLX, denaturation of the proteins or proteolytic degradation in mouse serum did not account for this decrease, since miniantibodies incubated in mouse serum for 4 h at 37 °C did not loose biologic activity (data not shown). Rather, the short half-life is probably a consequence of the small size of the molecule, as was recently shown for other miniantibodies, which are almost quantitatively excreted via the kidneys with a half-life similar to that measured here [26].

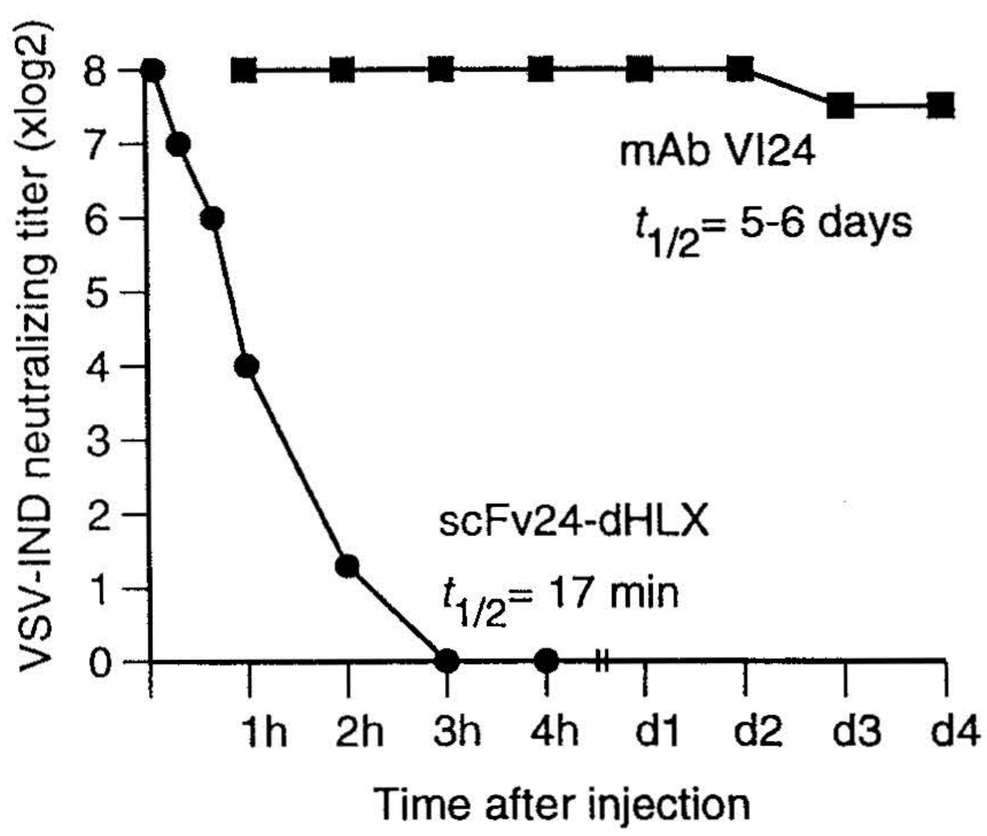

Figure 4. Blood levels of whole Ig and bivalent scFv24-dHLX in SCID mice. SCID mice were treated i.v. with 2 µg VI24 (filled squares) and 10 µg scFv24-dHLX (filled circles). VSV-neutralizing titers were determined in a standard VSV neutralization assay. The half-life in the circulation ($t_{1/2}$) is indicated. One of three similar experiments is shown.

4 Concluding remarks

The data presented in this study indicate that both monovalent scFv24 and bivalent scFv24-dHLX can neutralize VSV *in vitro* and that both reagents are able to protect against physiologically relevant virus doses independent of Fc-mediated functions. These results do not exclude that the Fc piece might contribute some help to protect, as suggested by data obtained with mouse hepatitis virus-specific $F(ab')_2$ fragments [27]. Nevertheless, our data are in contrast to other analyses demonstrating the involvement of the Fc portion in antibody-mediated protection [28–30]. In one example, mice developed lethal disease after intracerebral injection of pre-formed $F(ab')_2$-yellow fever virus complexes [30].

Our data document several new findings. To protect against VSV, covering up of determinants on the virus surface suffices and is critical for protection. Bivalency does not change the quality of VSV-specific binding, but it may be an important quantitative contributor to overcome certain affinity thresholds. Although monovalent and bivalent scFv fragments protect against VSV infection, if precomplexed in excess with the virus, they are not able to protect passively against virus in a conventional passive immunization setting, since their half-life is too short. Due to the fast disappearance from circulation and the absence of the Fc portion, scFv reagents are poor immunogens. Even after repeated scFv administrations, there is no scFv-specific antibody responses observed. Therefore, bivalent virus-specific scFv reagents might be useful for repeated local applications, as shown for recombinant RSV-specific Fab fragments, which exhibited a therapeutic effect in RSV-infected mice after repeated delivery of Fab fragments directly to the lung [17]. The half-life of non-glycosylated proteins is, above all, dependent upon their molecular weight. Recent progress in engineering tetravalent miniantibodies [31] should thus be an important step in the direction of passive immunization against viral disease with recombinant antibody fragments.

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