Recombinant Diabody-Based Immunocapture Enzyme-Linked Immunosorbent Assay for Quantification of Rabies Virus Glycoprotein

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The potency of rabies vaccines, determined using the NIH mouse protection test, can be directly correlated to the amount of rabies virus glycoprotein (RV GP) present in the vaccine. In an effort to develop a simple and sensitive enzyme-linked immunosorbent assay (ELISA) using recombinant diabody for quantification of RV GP, the variable heavy (VH) and light chain (VL) domains of an RV GP-specific human monoclonal antibody (MAb) secreted by a human × mouse heterohybridoma (human MAb R16E5) was amplified, linked using splicing by overlap extension PCR (SOE PCR), and expressed as a recombinant diabody (D06) in the pET28a bacterial expression system. The diabody D06 was purified by immobilized metal affinity chromatography on a nickel-nitrilotriacetic acid (NTA) agarose column and characterized. The purified diabody was used in combination with a well-characterized RV GP-specific mouse MAb, MA84, to develop an immunocapture ELISA (IC-ELISA) for the quantification of RV GP in human rabies vaccine preparations. The maximum detection limit of the IC-ELISA using the M5B4-D06 combination was up to 31.25 ng/ml of RV GP. The specificity of the diabody was established by its nonreactivity toward other human viral antigens as determined by ELISA and toward RV GP as determined by immunoblot transfer assay and competitive ELISA with the parent human MAb R16E5 and MAb M5B4. The adjusted \( r^2 \) value obtained by the regression through the origin model was 0.902, and the equation for predicted potency values for M5B4-D06-based IC-ELISA and MAb M5B4 IC-ELISA were 0.5651 and 0.8044, respectively, where \( x \) is the estimate of RV GP from the IC-ELISA in micrograms. Analysis of variance (ANOVA) results showed the estimates of the two methods differed significantly (\( P < 0.001 \)), while the predicted potencies by the two tests did not differ significantly (\( P > 0.05 \)). The IC-ELISA can be readily adapted to measure the RV GP content in purified antigen, and a vaccine can be formulated based on the estimated GP.

Rabies is a fatal viral infection of the nervous system affecting all mammals, including humans through bite wounds from a rabid animal, which can be prevented by vaccination coupled with administration of anti-rabies virus serum (6, 11). Rabies transmission from nonbite exposures is rare. Scratches, abrasions, open wounds, or mucous membranes contaminated with saliva or other potentially infectious material (such as brain tissue) from a rabid animal constitute nonbite exposures. Occasionally reports of nonbite exposure are such that postexposure prophylaxis is given. Inhalation of aerosolized rabies virus is also a potential nonbite route of exposure, but with the exception of laboratory workers, most people are unlikely to encounter an aerosol version of the rabies virus (5). Organ transplantsations have also been credited with nonbite transmissions of rabies from human to human (3). Despite significant scientific progress, rabies remains an important zoonotic disease globally. Annually, 20,000 deaths are reported in India, making rabies one of the major causes of human mortality (21). Vaccination is therefore considered one of the most viable and important methods for the prevention of rabies by way of preexposure prophylaxis in high-risk groups, postexposure prophylaxis in contact groups, and preexposure prophylaxis in pet animals that are at risk due to possible contacts with rabid animals. The most cost-effective means of prevention and control of rabies in humans is by eliminating rabies in dogs and other susceptible animals through vaccination.

The NIH mouse protection test is an in vivo potency test that has been used widely by all manufacturers of rabies vaccines. The role of different immunological parameters and the presence of virus-neutralizing antibodies are not well established because of a weak correlation between the NIH potency test results and immunogenicity when vaccines containing different strains of rabies virus were tested (2). Furthermore, this method is time-consuming and expensive, requires a large number of animals, and involves the use of live rabies virus. As a result, there is increased exposure in human beings to live and virulent rabies strains. The NIH test also requires a secure biosafety level 3 (BSL-3) facility for housing and challenging the experimental animals. Therefore, for both practical and ethical reasons, replacement of this test by more rapid and reliable in vitro methods is highly desirable. Based on the fact that the rabies virus glycoprotein (RV GP) is the antigen responsible for inducing virus-neutralizing
antibodies and conferring protection against a lethal intracerebral challenge, it has been suggested that the antigenicity of the rabies vaccines could be evaluated by titration of the RV GP (17).

Though some laboratories have used enzyme-linked immunosorbent assay (ELISA) to assess RV GP content for determination of the potencies of inactivated vaccines, variable correlation between ELISA and the NIH test has been reported. Essentially, all these ELISAs incorporate the use of either polyclonal antibodies or hybridoma-derived monoclonal antibodies (MAbs). Although MAbs offer substantial advantages with respect to potency, reproducibility, and freedom from contaminants (4), they are difficult to prepare in a quality-assured manner.

Recombinant DNA technology has been used to a great extent in the expression of antibodies/antibody fragments (2). Antibody fragments can be readily produced from the genes encoding antibody variable domains, which can be derived either from hybridomas (19) or from bacteriophage displaying antibody fragments (16). Diabodies are bivalent or bispecific antibody fragments generated by the dimerization of variable heavy (VH)-light chain (VL) fragments (10) as a result of reduction in the size of the linker between variable light and variable heavy chains (1), and these antibodies have many practical applications, including immunooassay and therapy.

We describe for the first time the use of a recombinant diabody in the development of an ELISA for quantification of RV GP content in human rabies vaccines incorporating the PV strain of rabies virus and its comparison with the NIH mouse protection test.

MATERIALS AND METHODS

Cells and heterohybridoma. The human × mouse heterohybridoma cell line formed by fusion between primary human peripheral blood B cells from a donor immunized with a human rabies vaccine (PV strain; Abhayrab) and a heteromyeloma cell line, K6H6/B5, secreting an RV GP-specific human MAB of the IgG1 isotype (human MAb R16E5) obtained from the hybridoma laboratory Indian Immunologicals Limited (IIL), Hyderabad, India, was used for amplification of variable light and variable heavy chain genes. A mouse neuroblastoma (Neuro-2a) cell line procured from ATCC and maintained in the cell culture laboratory IIL, Hyderabad, was used to carry out the indirect fluorescent antibody test (IFAT).

Bacterial strains, vectors, and chemicals. The bacterial strain Escherichia coli BL21(DE3) (Invitrogen) was used for the propagation of plasmids and overexpression of protein. The bacterial expression vector pET28a, used for cloning the bacterial strain BL21(DE3) (Invitrogen) was used for the propagation of plasmids and overexpression of protein. The bacterial expression vector pET28a, used for cloning the bacterial strain BL21(DE3) (Invitrogen) was used for the propagation of plasmids and overexpression of protein. The bacterial expression vector pET28a, used for cloning the bacterial strain BL21(DE3) (Invitrogen) was used for the propagation of plasmids and overexpression of protein. The bacterial expression vector pET28a, used for cloning the bacterial strain BL21(DE3) (Invitrogen) was used for the propagation of plasmids and overexpression of protein.

Isolation of total RNA and cDNA synthesis. The total RNA isolated from the heterohybridoma cell line (1 × 10^6 cells) using the TRIzol reagent (Invitrogen) was resuspended in diethyl pyrocarbonate (DEPC)-treated water and quantified using a Biophotometer instrument (Eppendorf, Germany). The cDNA was synthesized using random hexamers and a Thermoscript reverse transcriptase (RT)-PCR kit (Invitrogen) according to the manufacturer’s instructions. The cDNA was stored at −20°C until further use.

Amplification of variable domains, assembly, and cloning of diabody. The cDNAs encoding the antibody variable domains (VH and VL) were PCR amplified using universal primers (15). The variable regions were assembled using splicing by overlap extension (SOE) PCR (Table 1). The 711-bp-long PCR product was purified and cloned into the pCR2.1 TOPO TA vector to obtain pCRdia, and the PCR product was transformed into E. coli competent cells and plated on LB agar supplemented with 50 µg/ml kanamycin (LB-Kan). The plates were incubated overnight at 37°C. Selected clones were grown overnight in LB-Kan medium, and pure plasmid DNA was isolated using the Qiagen (Germany) Miniprep kit according to the manufacturer’s instructions. The plasmid DNA was purified, and the sequence was verified by automated cycle sequencing, and the plasmid was stored at −20°C until further use.

Expression and purification of the diabody. The pET28aRD was transformed into E. coli BL21(DE3), plated on LB-Kan, and incubated overnight at 37°C. A single colony of E. coli BL21(DE3) containing pET28aRD was inoculated in LB-Kan and grown overnight in an orbital shaker at 30°C at 200 rpm. The overnight culture was diluted 40 times in fresh LB-Kan and grown at 37°C at 200 rpm until the culture reached an optical density (OD) of 0.8 to 0.9 at 600 nm. The culture was induced with 1 mM isopropylthio-β-D-galactopyranoside (IPTG) by incubation at 28°C for 4 h. The bacterial pellet was collected by centrifugation at 5,000 × g for 20 min at 4°C.

Purification of diabody by IMAC. The bacterial pellet was resuspended in lysis buffer (50 mM Tris–HCl, 155 mM NaCl, pH 7.6) to prepare a 10% (wt/vol) suspension. Lysozyme was added to a final concentration of 50 µg/ml of lysate and incubated overnight at −20°C. The sample was subjected to sonication and centrifuged at 9,200 × g for 30 min at 4°C. The pellet was discarded, and the supernatant was subjected to immobilized metal affinity chromatography (IMAC).

An IMAC column (5-mL volume) was equilibrated with 10 column volumes of 50 mM Tris–HCl, 155 mM NaCl, pH 7.6 (equilibration buffer). The supernatant was loaded onto the column at a flow rate of 1 ml/min and washed with 20 column volumes of washing buffer (equilibration buffer with 30 mM imidazole, pH 7.6). Bound diabody was eluted with 5 column volumes of elution buffer containing equilibration buffer with 300 mM imidazole, pH 7.6, as 1-mL fractions. All the eluted fractions were analyzed by SDS–PAGE and immunoblotting. Fractions containing the recombinant diabody were pooled and dialyzed against phosphate-buffered saline (PBS), and the protein concentration was determined by the bicinchoninic acid (BCA) method before storage at −20°C until further use.

Characterization of the diabody. (i) Detection of diabody by SDS–PAGE and immunoblot analysis. The purified diabody was electrophoresed by SDS–PAGE (15% gel) and visualized using a silver staining reagent (Bio-Rad) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-C; GE Health care), using a transblot apparatus (Bio-Rad), following the manufacturer’s instructions. The blot was probed with an anti-His MAb probe (Pierce) and developed using 0.05% 3,3′,5,5′-tetrabromo-2,4-diaminobenzidine (TMB) (Sigma) and 0.03% hydrogen peroxide in PBS.

(ii) Immunocapture ELISA for determination of sensitivity of the diabody against RV. Titration of diabody D06 (450 µg/ml) at different concentrations (1:200 to 1:2,400) was performed against different concentrations of RV (1 µg/ml to 1.95 ng/ml) in a sandwich ELISA as described previously by Nagarajan et al. (18) with a few modifications wherein the diabody D06 at different concentration was used for detection. The binding of the diabody with RV GP was detected by addition of anti-His probe followed by 3,3′,5,5′-tetramethylbenzidine (TMB). The plate was incubated at 37°C for 10 min, and the reaction was stopped by addition of 1.25 M H2SO4. The absorbance was measured at 450 nm using a microplate reader (Bio-Tek).

(iii) Demonstration of RV-GP-specific activity of the diabody. (a) Immunoblot transfer assay with rabies virus antigen. Zonal purified whole virus (PV strain) antigen (4 µg) was fractionated by SDS–PAGE using a 10% gel under native condition. The resolved proteins were transferred onto a PVDF membrane (Hybond-C; GE Health care) and blocked with 2% nonfat milk powder (Difco) in PBS for 1 h at room temperature. The blot was washed thrice with PBS containing 0.05% Tween 20 (PBS-T), followed by probing of the membrane with diabody D06 (25 µg/ml) for 1 h at room temperature. A rabies virus glycoprotein (RV GP)-specific mouse Mab, MSB4 (18), and the parent human Mab, RI6E5, were used as a positive control, and a rabies virus nucleoprotein (NP)-specific Mab, NSG4, was used as a negative control. The immunoreactivity with RV GP was detected by probing the blot with a His probe (Pierce) at a dilution of 1:5,000 followed by staining with DAB.
reaction was stopped by addition of 100 µl of TMB (Sigma) was added. The absorbance was measured at the 450-nm wavelength using a microplate reader (Bio-Tek). The assay was performed in triplicate. The RV GP content was also measured at the 450-nm wavelength using a microtiter plate reader (Bio-Tek). The experiment was performed in triplicate. Immunocapture ELISA for quantification of rabies virus glycoprotein in human rabies vaccine. IC-ELISA was performed to quantify the RV GP content in rabies vaccine formulations, according to the method described by Nagarajan et al. (18), with a few modifications wherein the diabody (450 ng/well) was used for detection. Briefly, the ELISA plate was coated with PBS-T and dried. The diabody at a concentration of 450 ng/100 µl, and HBsAg (32 µg/ml), HAV (100 IU/ml) and MAb M5B4 (156 ng/well) were added to each well. The plates were incubated at 37°C for 1 h, washed with PBS-T thrice, and dried by flicking. Anti-His probe was added, followed by incubation for 1 h at 37°C. The plate was developed using hydrogen peroxide-activated TMB. The reaction was stopped by addition of 1.25 M H2SO4, and the absorbance was measured at 450 nm using a microplate reader (Bio-Tek).

(b) Competitive ELISA. A competitive ELISA was performed to identify the specificity of the diabody D06 for RV GP. A microtiter plate was coated with 100 ng/well of purified RV (PV) antigen in 50 mM carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed thrice with PBS-T and blocked with 1% bovine gelatin (Sigma) in PBS-T, followed by washing with PBS-T to remove the excess gelatin. Diabody (500 ng/100 µl) was added by serial dilution and incubated at 37°C for 1 h. E. coli lysate (vol/vol) was used as a negative control. An RV GP-specific MAb, MSB4 (156 ng/well), and the human MAb R16E5 (200 ng/well) were added to each well containing diabody and negative control. An RV GP-specific MAb, M5B4 (156 ng/well), and the human diabody and monoclonal antibodies M5B4, specific for RV-GP, and 1F6, specific for HBsAg (unpublished), and polyclonal mice sera raised against HAV and CHIKV at concentrations of 156 ng/well, 140 ng/well, 300 ng/well, and 300 ng/well, respectively, and incubated overnight at 4°C. The wells were washed thrice with PBS-T, and the unreacted sites were blocked with 1% bovine gelatin in PBS-T by incubation at 37°C for 1 h. The plate was washed thrice with PBS-T and incubated with mouse anti-His IgG-fluorescein isothiocyanate (FITC) conjugate at a concentration of 1 µg/5 ml (AnaSpec) at 37°C for 45 min. The plate was washed five times with PBS-T, and 100 µl of TMB (Sigma) was added. The reaction was stopped by addition of 100 µl of 1.25 M H2SO4 to each well, and absorbance was read at 450 nm using a microplate reader (Bio-Tek). The specificity of the diabody toward antigenic site III of PV GP was determined, following a method described by Nagarajan et al. (18).

(iv) IPAT. The diabody (500 ng/100 µl) was used to probe the unified RV (PV strain)-infected and uninfected mouse neuroblastoma (Neuro-2a) monolayer (6 × 10^6/well) in a 96-well tissue culture plate (Nunc, Denmark) and incubated at 37°C for 45 min in a CO2 incubator. The plate was washed with PBS, pH 7.4, and incubated with mouse anti-His IgG-fluorescein isothiocyanate (FITC) conjugate at a concentration of 1 µg/5 ml (AnaSpec) at 37°C for 45 min in a CO2 incubator. The plate was washed with PBS to remove the excess conjugate and observed under a fluorescence microscope (Olympus, Japan). The RV GP-specific MAb MSB4 was detected using the diabody followed by the addition of anti-His probe. The plate was developed with TMB at room temperature for 10 min. The reaction was stopped by addition of 1.25 M H2SO4, and the absorbance was measured at the 450-nm wavelength using a microtiter plate reader (Bio-Tek). The assay was performed in triplicate. The RV GP content was also...
estimated by MAb M5B4 IC-ELISA, previously described by Nagarajan et al. (18), using the reference standard vaccine. The RV GP content was estimated using the formula RV GP estimate (micrograms/dose) = (X \times Z \times A \times 10)/Y, where X is the optical density of the sample, Y is the optical density of the IRS equivalent to twice the mean of the optical density of the negative control, Z is the reciprocal of the endpoint dilution, and A is the GP estimate of Y in nanograms.

NIH potency test on rabies vaccine formulations. The NIH potency test was carried out on the different rabies vaccine formulations in mice using standard procedures (23).

Statistical analysis. The estimates of RV GP antigen and predicted potency derived from the IC-ELISA using the MAb M5B4 (18) and diabody D06 were compared with the in vivo NIH potency results using ANOVA with the regression through the origin (RTO) model (22).

RESULTS

Assembly, cloning, and expression of diabody. The V_{H} and V_{L} domains of the antibody were PCR amplified from a human × mouse heterohybridoma secreting RV GP-specific human MAb (Fig. 1, lanes A and B). The amplified V_{H} and V_{L} chains were joined together with the 24-mer polynucleotide linker using SOE PCR, and the resultant 711-bp-long PCR product (Fig. 1, lane C) was cloned into the TOPO-TA vector to yield pCRdia. pCRdia was sequenced, and the entire sequence was submitted to the international ImmunoGeneTics information system (IMGT) for sequence verification. Sequence information revealed the presence of 363-bp-long V_{H}, 324-bp-long V_{L}, and a 24-bp-long linker region (Fig. 2).

The diabody gene was reamplified using primers containing EcoRI and NotI sites at the 5’ and 3’ ends, respectively, from pCRdia, gel purified, and cloned into pET28a to obtain pET28aRD to obtain pET28aRD (Fig. 1, lanes D and E). pET28aRD was transformed into BL21(DE3), and the diabody was expressed by induction with 1 mM IPTG. The cell pellet was lysed, and the cytoplasmic fraction was purified by IMAC. Analysis of the purified recombinant diabody by immunoblotting indicated the presence of an ~30-kDa band (Fig. 3). The yield of diabody was ~5 mg per 10-liter culture.

Characterization of diabody. (i) Determination of sensitivity of the diabody using IC-ELISA. A checkerboard titration was performed to determine the optimal concentration of diabody D06 to be used in IC-ELISA for quantification of RV GP (Fig. 4). The highest and lowest detection limits for RV GP under a linear detection range were determined using purified RV (PV strain). The optimal dilution of D06 required was 450 ng.

(ii) Reactivity of diabody with rabies virus glycoprotein. Immunoblotting of the diabody D06 against the Pasteur rabies virus (PV) structural proteins resolved in a 10% gel by nonreducing SDS-PAGE clearly indicated the binding of the diabody, parent human MAb R16E5, and MAb M5B4 to an ~66-kDa protein which corresponded to PV GP (Fig. 5). The PV NP-specific MAb N5G4 was used as a negative control, and it bound to an ~55-kDa protein corresponding to PV NP.

(iii) Competitive ELISA. Competitive ELISA was performed to determine the competition between diabody D06, parent MAb R16E5, and MAb M5B4 for an ~66-kDa protein which corresponded to PV GP. Competition could be seen when the constant amount of MAb R16E5 was allowed to compete with various amounts of

FIG. 1. Agarose gel electrophoresis analysis of PCR-amplified products from human × mouse heterohybridoma. Lanes M show the DNA ladder, and lanes A, B, and C show the variable heavy and variable light chain genes and assembled PCR products. Lanes D to G show the recombinant expression cassette after EcoRI and NotI digestion, wherein lanes D and E show release of ~711-bp product.

FIG. 2. Amino acid sequence of anti-rabies virus human diabody containing V_{L}, linker peptide, and V_{H}. The linker peptide is marked in italics.

FIG. 3. Detection of recombinant diabody by Immunoblotting. SDS-PAGE was performed with a 12% gel. Lane M shows the protein molecular size standard (New England Biolabs), and lane 1 shows purified diabody. The blot was transferred onto a PVDF membrane, probed with anti-His probe, and developed by using the DAB substrate. Lane M shows prestained protein molecular size markers; lane 1 shows a soluble fraction. The diabody (30 kDa) is denoted by an arrow.
the diabody. A gradual increase in OD values following the dilution of the diabody indicated that the diabody competed with MAb M5B4 for antigenic site III on RV GP (Fig. 6).

(iv) IFAT using diabody. The binding specificity of diabody for RV GP was determined by IFAT using unfixed, noninfected control and PV-infected Neuro-2a cells. Typical membrane fluorescence could be seen both with the diabody and with MAb M5B4 (Fig. 7). No fluorescence could be seen in uninfected control Neuro-2a cells.

(v) Specificity of diabody. Sandwich ELISA performed to determine the binding specificity of diabody D06 clearly showed reactivity with RV and not with other viruses, such as HAV, hepatitis B virus (HBV), and CHIKV (Table 2), indicating that diabody D06 can be used to detect RV without any cross-reaction with other virus antigens.

Estimation of PV-GP in various vaccine preparations. PV GP content was estimated in 65 batches of experimental human rabies vaccine preparations using the IC-ELISA, and the estimates were compared to the NIH potency values of those respective batches. ANOVA with the regression through the origin (RTO) model was performed to compare the potency estimates derived by M5B4-D06 IC-ELISA and MAb M5B4 IC-ELISA previously described by Nagarajan et al. (18). Regression analysis was performed using the data analysis program in Microsoft Excel 2003 to compare the potency estimates derived by M5B4D06 IC-ELISA and MAb M5B4 IC-ELISA with the NIH estimate. The adjusted $r^2$ value obtained was 0.902, and the equation for predicted potency values for M5B4-D06 based IC-ELISA and MAb M5B4 IC-ELISA were $0.5651x$ and $0.8044x$, respectively, where $x$ is the estimate of RV GP determined by the IC-ELISA in $\mu g$ (Fig. 8). ANOVA results showed that the estimates by the two methods compared differed highly significantly ($P < 0.001$), while the predicted potencies determined by the two tests did not differ significantly ($P > 0.05$).

**DISCUSSION**

Rabies endemicity in many developing countries is responsible for human deaths. The annual number of human deaths worldwide caused by rabies is estimated to be between 40,000 and 70,000 in Africa and Asia, where rabies is endemic (25), and India ranks among the highest with 20,000 human deaths (21). The disease can be prevented by the timely administration of vaccine or a combination of vaccine and RV immunoglobulins. Vaccine manufacturers around the world determine the potency of the rabies vaccines using the in vivo mouse protection test (23), which is time-consuming and expensive and requires the use of a large number of mice and virulent rabies virus for challenge in a biosafety level 3 (BSL-3) facility. The test, apart from being labor-intensive, time-consuming, and expensive, also suffers from poor intra- and interlaboratory
reproducibility, thus making it a prime target for replacement with easier *in vitro* tests that allow the accurate quantification of the rabies glycoprotein (20). In this article, we describe the expression, purification, and immunological characterization of a recombinant diabody followed by its application in development of an IC-ELISA for the quantification of RV GP in human rabies vaccines.

The study describes the construction and characterization of a recombinant diabody and its further development into a reagent for use in an IC-ELISA format to quantify RV-GP. Total RNA was isolated from a heterohybridoma, and the VH and VL fragments were amplified using universal primers and assembled into a diabody using a reduced peptide linker. The resultant PCR product was cloned into a bacterial expression vector, pET28A, and expressed in *E. coli* under the control of a T7 RNA polymerase promoter as an ~30-kDa soluble molecule. The diabody was purified by IMAC. Purification of the bacterial lysate resulted in a yield of ~0.5 mg of purified diabody/liter of culture with a homogeneity of ~85%. The ease of purification reiterated the fact that expression of a functional recombinant antibody in bacteria offered many advantages over the maintenance of a hybridoma cell line, which included minimal batch-to-batch variation, ease of scale-up, etc., at a reasonable cost.

The RV GP has been shown to induce neutralizing antibodies and confer protection against lethal rabies virus challenge. Thus, much of the effort has been directed toward the development of *in vitro* methods like ELISA, which is capable of estimating GP content in rabies vaccine preparations. The conventional/recombinant antibodies used in the assay should be able to recognize the highly immunogenic, natively folded RV GP present on the virus particle and should circumvent problems associated with the estimation of the poorly immunogenic soluble form of RV GP that could lead to the overestimation of the antigen, resulting in lower vaccine potency.

In order to demonstrate the binding of the diabody to the

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**FIG. 6.** Competitive ELISA using the diabody and the RV GP-specific parent human MAb R16E5 and mouse MAb M5B4. Complete or broken lines with squares show the result of competitive ELISA with diabody D06 and parent R16E5 with purified rabies virus antigen or *E. coli* lysate, respectively. Complete or broken lines with circles show the result of competitive ELISA with diabody D06 and MAb M5B4 with purified rabies virus antigen or *E. coli* lysate, respectively.

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**FIG. 7.** Demonstration of the specificity of recombinant human diabody expressed in *E. coli* cells for RV GP by IFAT. (a) Detection with anti-mouse IgG–FITC conjugate. (b) Detection with mouse anti-His IgG–FITC conjugate. (c) Uninfected-cell control.
native RV GP, rabies-infected Neuro-2a cells were allowed to react with the diabody. The appearance of specific membrane fluorescence indicated that the antibody bound to the native form of RV GP. The diabody also bound well to the RV GP, as demonstrated by Western blot analysis. Further, the diabody competed with MAb M5B4 for binding to RV GP in a concentration-dependent manner, suggesting that diabody and M5B4 bind to the same epitope. A specificity study showed that the diabody reacted only with RV but not with other viruses, as indicated by sandwich ELISA. Diabody holds promise in quantification of the RV GP in vaccine preparations, which correlated well with the NIH mouse potency. Various vaccine batches tested by diabody-based IC-ELISA showed a good correlation with the NIH mouse potency studies, as seen with MAb M5B4-based IC-ELISA (18, 24).

| Antibody                        | Reactivity against | Rabies virus | Hepatitis A virus | Hepatitis B virus | Chikungunya virus |
|---------------------------------|--------------------|--------------|-------------------|-------------------|-------------------|
| Diabody D06                    | +                  | +            | –                 | –                 | –                 |
| MAb M5B4                       | +                  | –            | –                 | –                 | –                 |
| Parent human MAb R16E5         | +                  | –            | –                 | –                 | –                 |
| MAb 1F6                        | –                  | –            | +                 | –                 | –                 |
| Mouse serum HAV                | –                  | +            | –                 | –                 | –                 |
| Mouse serum CHIKV              | –                  | –            | –                 | +                 | –                 |

*+, positive reactivity; –, no reactivity.

**FIG. 8.** (a) Line fit plot for regression through origin analysis for GP content estimated by MAb M5B4-D06 IC-ELISA and the actual NIH potency value (IU). (b) Line fit plot for regression through origin analysis for GP content estimated by MAb M5B4 IC-ELISA and the actual NIH potency value (IU).
Quantification of RV GP using diabody-based IC-ELISA provides exact information on the natively folded RV GP antigen in vaccine preparations and in-process control samples, which enables reliable estimation of RV GP. The method described in this article, using diabody, provides a simple, novel, and efficient option for quantification of RV GP antigen in vaccine preparations without a loss of antigen and aids in the manufacture of good-quality vaccines, which can reduce the cost and make the vaccine affordable in developing countries, where rabies prevention and control are a challenge. The diabody-based IC-ELISA could replace the MAb-based IC-ELISA due to better reagent stability and ease of production.

Conclusions. A diabody D06 consisting of the V_H and V_L portions of a parent human × mouse heterohybridoma human MAb, R16E5, was constructed using standard procedures. The sensitivity and specificity of the diabody were established for rabies virus glycoprotein. An immunocapture ELISA was standardized using the diabody D06 to estimate the glycoprotein content of the human rabies vaccine containing Pasteur virus. The IC-ELISA was compared with another procedure, described earlier by Nagarajan et al. (18), and with an in vivo NIH mouse protection test. Correlation could be established between the IC-ELISA using MSB4-D06 and the mouse protection test. This diabody-based ELISA can be used as an alternative for quantification of RV GP in purified RV antigens or vaccines before or after blending, respectively.

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