Rabbit anti-rabies immunoglobulins production and evaluation

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Abstract. Due to the disadvantages of human and equine rabies immunoglobulin, it is necessary to develop a substitute for HRIG and ERIG, especially for those people living in the developing countries. Because of higher affinity and lower immunogenicity of rabbit’s immunoglobulins, anti-rabies immunoglobulins specific to rabies virus were produced in rabbits as a bioreactor, and had been characterized by ELISA, affinity assay, immunofluorescence assay (IFA), immunocytochemistry, rapid fluorescent focus inhibition test (RFFIT). ELISA, affinity assay and IFA showed that rabbit RIG (RRIG) bound specifically to rabies virions. RFFIT result showed that RRIG has neutralization activity. This result was confirmed in vivo in a Kunming mouse challenge model and the protection rate of the treatment with RRIG was higher (25%) than that offered by HRIG when mice were challenged with a lethal RV dose. Our results demonstrate that RRIG is safe and efficacious as a candidate drug to replace rabies immunoglobulin in post-exposure prophylaxis.

INTRODUCTION

Rabies, a disease of the central nervous system, is caused by a virus belonging to the family Rhabdoviridae and genus Lyssavirus. It is a major disease of public health importance affecting human beings as well as domestic and wild animals in many parts of the world, especially in developing countries, where it is endemic among dogs (Meslin et al., 1994; Dietzschold et al., 1996). It is estimated that at least 14 million people each year take rabies prophylaxis following exposure to rabid animals in the world (Petricciani, 1993.) and that 55,000 people die from rabies per year (Meslin et al., 2001). According to the category of the exposure as defined by the World Health Organization (WHO), the most severe cases, Category III, require wound cleaning, rabies vaccination, and direct wound infiltration with rabies immunoglobulin (RIG) and where possible, observation of the biting animal for a period of 10 days if it does not already display clinical symptoms of rabies (Rupprecht, 2004; WHO, 2005). Infiltration of RIGs into the wound is essential in the management of severe bites to provide passive antibody protection during the first 1–2 weeks while the body develops its own immune response to vaccination (Quiambao et al., 2008). WHO recommends the use of human RIG (HRIG) or equine (ERIG) in category III exposures (WHO, 2005). However, when HRIG is not available or accessible, ERIG or heat-treated equine rabies immunoglobulin (pERIG) must be applied. As availability of HRIG is
constrained by limited production capacities imposed when using human plasma as the immunoglobulin source, bite victims in highly endemic countries are more likely to receive ERIG or pERIG. Nevertheless, the most common reasons for this shortage of HRIG are the cost and/or blood borne infectious diseases. ERIG is produced but its quality falls far short of the requirement (WHO, 1984; 1992). In a recent study, one batch of pERIG was shown to be poorly protective in an animal model (Syrian hamster) challenged with a virulent North American rabies virus strain (Hanlon et al., 2001). Therefore, it is necessarily required to develop a substitute for HRIG and ERIG.

Alternative sources to human and equine anti-rabies for treatment of rabies should be considered and will include human monoclonal antibodies, human recombinant antibodies (Sloan et al., 2007), and other animal's antibodies, such as those of ovine (Redwan et al., 2009). Nowadays rabbit antithymocyte globulin produced against homogenates of thymic tissue and anti-T lymphocyte globulins produced against the human T cell line are widely used in clinical therapy (Lytton et al., 2007). Many papers also reported that rabbit anti-thymocyte globulins induction therapy is as efficient and as safe as induction with anti-CD25 monoclonal antibodies (Kamar et al., 2005; Klem, 2009). Redwan et al. (2009) reported that rabbit IgG has low immunogenic effect than human, horse and sheep antibodies when similar concentration of either horse, human, sheep, rabbit, or camel immunoglobulins (IgGs) were injected into the mouse. Moreover, the use of rabbit antibodies has been shown to be advantageous in many ways. First, direct comparisons of rabbit and mouse monoclonal antibodies directed against human melanoma cells have shown that rabbit and mouse antibodies recognize different epitopes; second, rabbit antibodies are generally of higher affinity than mouse antibodies (Patent No. 5675063,1997). All previous studies showed that rabbit IgGs may be applied to disease therapy. In this study, we will investigate the feasibility of using the rabbit as host to produce the anti-rabies globulins.

MATERIALS AND METHODS

Rabies strains, cells and animals
Rabies virus strain CTN (which is 83.2% - 96.8% and 90.0% -97.4% homologous to street strain in nucleic acid and amino acid sequences, respectively), was provided by Wuhan Institute of Virology, Chinese Academy of Sciences. Rabies virus strain CVS-11, CVS-24, Flury and BHK-21 cells were from the Veterinary Institute of the Academy of Military Medical Sciences, China. BHK-21 cells were cultured in DMEM (Gibico, US) supplemented with 10% FBS. Cell lines were maintained at 37°C under an atmosphere of 5% CO₂.

Kunming mice (10 g – 12 g) were obtained from the Experimental Animal Center of the Academy of Military Medical Sciences of China and all breeding and experiments were approved by the Veterinary Institute of the Academy of Military Medical Sciences animal Ethics Committee [Project numbers SYXK (ARMY) 2009- 045].

Rabbit Immunization
Two New Zealand rabbits were immunized with inactivated purified virus CTN strain. For the initial immunization, rabbits were immunized with inactivated purified virus protein (50 µg per rabbit) emulsified in Freund's complete adjuvant. Subsequent booster shots were given every 2 weeks with immunogens (50 µg per rabbit) in Freund's incomplete adjuvant (Huang et al., 2007). After four immunizations, blood samples were collected and taken to determine antibody titer by ELISA, preimmunized samples were used as a negative control. These rabbits were boosted intravenously with 100 µg of immunogens 7 days before they had been sacrificed.
Rabbit anti-rabies IgG purification
Rabbit anti-rabies IgG was purified according to previous protocol (Hickey et al., 1991). In brief, the clarified serum was mixed with an equivalent volume of veronal buffered saline (pH 7.2). Then 15mg/ml serum of silicon dioxide powder was added and stirred for 30 min at room temperature (25ºC–30ºC). The mixture was centrifuged at 10,000 g for 30 min. The supernatant was purified by protein G chromatography, according to manufacturer’s protocols (GE, USA).

SDS-PAGE analysis
The concentration of the purified sample was determined by spectrophotometer at 280 nm. The same amount of IgG of each preparation was loaded in SDS-polyacrylamide gels (SDS-PAGE), and electrophoretic separation was performed under reducing conditions as previously described (Redwan, 2006). Proteins were stained with coomassie Brilliant Blue R-250. Molecular mass standards were run in parallel.

Reactivity of rabbit anti-rabies IgGs with rabies virus protein in indirect ELISA
Ninety-six-well plates (Costar, USA) were coated with the purified rabies virus protein diluted in 50mM carbonate saline (pH 9.6) and incubated overnight at 4ºC. As a negative control, DMEM medium which produced rabies antigens was coated. Plates were blocked by incubating for 3 h at 37º using 1% BSA in PBS followed by three washings in 0.5% PBS-Tween 20. Serially diluted IgGs in PBS (100µl) were added to each well in triplicate and incubated for 1 h at 37º followed by three more washings in 0.5% PBS-Tween 20. Secondary antibody, goat anti-rabbit IgG horseradish peroxidase (Santa Cruz) was added at 1:5000 and the samples were incubated for 1 h at room temperature followed by three more washings in PBS-0.5% Tween 20. One hundred µl of the mixture of H₂O₂ and 3,3’5,5’-tetramethylbenzidine substrate (TMB; Thermo scientific, USA) was added to develop color reaction. The reaction was stopped by adding 50µl of 2M H₂SO₄. Plates were read for optical density at 450 nm with a reference wavelength of 630nm on a multiskan spectrum (Thermo labsystems, USA). The positive thresholds (cut-off value) were defined as 2.1 the mean of standard negative values (He et al., 2006).

Western blot analysis
To further examine whether the rabbit anti-rabies IgG recognize the protein of rabies virus protein, Western blot analysis was performed under denaturing conditions. Rabies virus protein was run on 12% SDS-PAGE, an equal amount of bovine serum albumin (BSA; Sigma–Aldrich) was used as a negative control. Then the proteins were electrotransferred onto a nitrocellulose membrane (Amersham Biosciences, UK) and blocked with 5% non-fat dry milk in PBS. After overnight blocking at 4ºC with PBS containing 1% BSA (PBSA), the membranes were incubated with purified rabbit anti-rabies IgGs (1:2000) for 2 h at room temperature. After 3 washes with 0.05% Tween-20 in PBS (PBST), the membrane was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:2000 dilution; Santa Cruz) for 1 h at room temperature, then washed again. Antibody binding was visualized by addition of the mixture of H₂O₂ and diaminobenzidine substrate (DAB; Sigma–Aldrich). After incubation for 15min at 37ºC, the reaction was stopped by addition of H₂O.

Affinity analysis by surface plasmon resonance (SPR)
Surface plasmon resonance (SPR) analysis was performed on a BIAcore 3000TM analytical system (Bakker et al., 2005). Rabies virus protein was diluted at 5µg/ml with acetate buffer (10 mM NaAc, pH 5.5, GE, USA) and immobilized on the surface of a CM5 sensor chip (GE, USA) to capture purified rabbit anti-rabies IgGs. Purified IgG was diluted in HBS-EP buffer (GE, USA) at concentrations ranging from 31.25 to 1000 nmol/L and performed at a constant flow rate of 30µl/min for 3min at 25ºC. The association time was 180 sec and the
The dissociation time was 600 sec, followed by regeneration with 10mM Glycine-HCl (GE, USA). The sensorgrams were evaluated using the BIAcore T100 evaluation software.

**Immunofluorescence assay (IFA)**

Binding of rabbit anti-rabies IgG to rabies virus infected cells was determined by IFA. Sub-confluent BHK-21 cells in 24-well microplates with slides were infected with Flury strain of rabies. After incubation for 24 h, diluted rabbit anti-rabies IgGs (1:100) were added to virus-infected BHK-21 cells. The uninfected BHK-21 cells were used as a negative control. After incubation at room temperature for 2 h, slides were washed 3 times with PBST, and FITC-conjugated anti-rabbit IgG was added at dilution of 1:50. Slides were washed again after 1 h incubation, cell nuclei were dyed with HOECHST 33342 solution (1:10000, Dojindo, Japan) at 1mg/ml for 5 min at 37ºC, and observed under fluorescence microscope at 400x magnification. Cells showing strong green fluorescence were recorded as positive.

**Immunocytochemistry**

The slides with BHK-21 cells infected with rabies Flury strain were used for immunocytochemistry. The slides were immobilized with ethanol. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide in methanol for 30 minutes. The uninfected BHK-21 cells were used as a negative control. The slides were then blocked with 10% goat serum for 15min and subsequently incubated with purified rabbit anti-rabies IgGs overnight at 4ºC. After being washed extensively, the slides were treated with biotinylated goat anti-rabbit antibody and then Streptavidin/HRP (SP-9001 Histostain TM-Plus Kit, Zymed, ZSGB-BIO) for 20 minutes each step. Reaction product was visualized with DAB at room temperature for 20 seconds. Counterstaining was performed with hematoxylin, and the sections were then dehydrated and mounted (Zou et al., 2006).

**Rapid fluorescent focus inhibition test (RFFIT)**

Neutralizing antibody titers against rabies virus were determined by RFFIT. In a 96-well plate (Costar, USA), the antibody was diluted two fold with DMEM that contained 10% FCS and placed in a well. The samples were set up in duplicates. CVS-11 (100 TCID<sub>50</sub>) was added to each well and incubated in a 5% CO<sub>2</sub> incubator at 37ºC for 90 min. Next, BSR cells were added to each well and incubated for 24 h. Finally, cells were fixed with 80% acetone and stained with FITC-conjugated anti-rabies N monoclonal antibody (Fujirebio Diagnostics, Inc., Malvern, PA) at 37ºC for 30 min, and observed under a fluorescence microscope. Average of duplicate samples was determined. The neutralizing antibody titer was calculated by Reed-Muench method. A neutralizing antibody titer of ≥0.5 IU/ml was defined adequate for protection against rabies (WHO, 1992; Shiota et al., 2009).

**In vivo Kunming mice challenge model**

A lethal animal model mimicking rabies exposure was used as described elsewhere. Briefly, Kunming mice (4 groups, 8 mice/group, 10-12 g) were infected with 100LD<sub>50</sub>/0.05 ml rabies virus CVS-24 strain (stocked in the Veterinary Institute of the Academy of Military Medical Sciences, China). Three hours later, the mice were vaccinated with rabies vaccine (Sanofi-Pasteur S.A.) except for group control. In addition, HRIG (Taibang Ltd, CN) at 20 IU/kg and RRIG at 20 IU/kg were administered. On day 7, the mice were vaccinated again except for group control. The mice were examined daily for clinical signs of rabies and death. The mice were maintained and evaluated up to day 28 after infection. The experiments that involved the use of rabies strain CVS-24 was performed in a BSL-3 laboratory. At necropsy, brain impressions were made and tested for rabies virus antigen by the direct fluorescent antibody test (Sloan et al., 2007; Redwan et al., 2009).
RESULTS

Generation and purification IgG against rabies virus protein
Antibody titer of rabbit blood sample was higher than 1:10000, which indicates a good response to the rabies virus. 100ml of blood samples were collected per rabbit. All sera were efficiently purified by protein G chromatography. The electrophoretic analysis of all preparation showed two distinct bands in reducing condition at 55 and 25 kD which correspond to IgG-heavy and light chains, respectively (Figure 1). The concentrations of purified IgGs were determined as 6.3 mg/ml, and 126 mg of IgGs that were purified from 100ml blood sera samples.

Western blot analysis
The binding specificity of the rabbit anti-rabies IgG against denatured rabies protein was determined by Western blot analysis (Figure 2). The data indicated that the rabbit anti-rabies IgG bind denatured rabies virus protein.

Reactivity of IgGs with rabies virus protein in indirect ELISA
To examine the reactivity of the rabbit anti-rabies IgGs with rabies virus protein, the indirect ELISA was performed with rabies virus protein. The titers of rabbit anti-rabies IgGs were higher than 1 : 179200 in indirect ELISA. The reactivity of purified rabbit anti-rabies IgGs with rabies protein is shown in Figure 3. Rabbit anti-rabies IgGs showed strong positive binding with rabies protein, compared to the DMEM medium.

Binding affinity between purified IgGs and rabies protein
The binding affinity between rabies protein and purified rabbit anti-rabies IgGs was analyzed by Biacore T100 (GE, USA). Rabbit anti-rabies IgGs bound to rabies virus protein with an affinity of 1.551x10^-8 M (Figure 4).

Figure 1. SDS-PAGE analysis of purified rabbit IgGs
The concentrations of purified IgGs were determined as 6.3mg/ml. Proteins were stained with Coomassie Brilliant Blue R-250. Estimated molecular masses are depicted in boxes on the left side represent the heavy (55 kD) and light (25 kD) chains of rabbit IgG, respectively. Lane 1, flowthrough of the purified IgG of immunized rabbit serum. Lane 2, non-purified IgG of immunized rabbit serum. Lane 3, IgG purified from control rabbit serum.

Figure 2. Western blot analysis of purified rabbit IgGs
Lane 1 represents the rabbit anti-rabies IgGs recognized the protein of rabies virus glycoprotein (65kD), while lane 2 was the rabbit anti-rabies IgGs with the protein of BSA.
Figure 3. Indirect ELISA analysis of rabbit anti-rabies IgGs with rabies virus protein
Serially diluted (1:100-1:179200) rabbit anti-rabies IgGs was tested against rabies virus protein by ELISA. DMEM medium which produced rabies antigens was used as a negative control (NC).

Figure 4. Biacore binding curves of purified rabbit anti-rabies IgGs with rabies virus protein
A concentration series from 31.25 to 1000 nM of purified rabbit anti-rabies IgGs was injected (associated for 180s and then dissociated over 600s). The affinity constant KD was determined as koff/kon.
Immunofluorescence assay (IFA)
IFA was performed to further analyze whether the rabbit anti-rabies IgGs recognized the protein in rabies-infected BHK-21 cells. Rabbit anti-rabies IgGs showed strong reactivity with rabies-infected cells, while not showing specific binding to the uninfected BHK-21 cells (Figure 5).

Immunocytochemistry
Immunocytochemistry result indicated that rabbit anti-rabies IgGs recognize rabies virus protein in fixed cells. The results showed that the cell was strongly stained with rabbit anti-rabies IgGs, while the uninfected BHK-21 cells were no stained with the anti-rabies IgGs (Figure 6).

Rapid fluorescent focus inhibition test (RFFIT)
Neutralizing antibody titer against rabies virus was 6.7 IU/ml determined by RFFIT. About 670 IU of purified rabbit anti-rabies IgGs were produced from two immunized rabbits. The titer could reach the titration value of 2.5 IU/mL which was believed by WHO expert committee to be adequate for providing protection immunity against rabies in human easily.

Activity identification in vivo
Neutralizing activity of RRIG was further confirmed in Kunming mice models. As summarized in Table 1. In group control, only 1 mouse (1/8) survived without vaccine and antibodies, indicating that the virus were lethal; in group vaccine, only 2 mice (2/8) survived, indicating that vaccine cannot protect all mice before generating enough antivirus antibodies to vaccine; in group HRIG plus vaccine, 5 (5/8) mice survived, indicating that HRIG plus vaccine can provide 62.5% protection; in group RRIG plus vaccine, 7 mice (7/8) survived, indicating that RRIG plus vaccine can provide 87.5% protection , which was 25% higher than HRIG with the equivalent IU/ml.

Figure 5. Detection of rabbit anti-rabies IgGs with RV-infected BHK-21 cells by immunofluorescence assay
The slides were observed by fluorescence microscopy (400x). A-1 and B-1, cell nuclei were dyed with HOECHST 33342 solution; A-2, RV-infected cells stained with rabbit anti-rabies IgGs; B-2, Uninfected BHK-21 cells stained with rabbit anti-rabies IgGs
Table 1. The survival rate after PEP

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<tr>
<td>Survival rate (%)</td>
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<td>25</td>
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</table>

DISCUSSION

According to WHO guidelines, category III exposure to rabies, which is defined as either single or multiple transdermal bites or contamination of mucous membranes with saliva of a rabid animal, requires rabies postexposure prophylaxis (PEP) (Dietzschold et al., 1996). Rabies PEP includes administration of both vaccine and anti-rabies immunoglobulin (RIG). At present, RIG for PEP mainly includes HRIG and ERIG, which are prepared from the serum samples of either rabies virus immune humans or horses. But HRIG is not widely available and it is costly. There is apprehension about the side-effects of ERIG, especially anaphylactic reactions (Redwan et al., 2009). It became also difficult to obtain ERIG because the major international supplier decided to discontinue the production of ERIG (WHO, 1984). Therefore, it is necessary to develop a substitute for HRIG and ERIG.

Many studies indicated that rabbit IgG has some advantages: lower immunogenicity (Redwan et al., 2009), higher affinity, and recognition of different epitopes (Patent No. 5675063, 1997). They are efficient and safe (Kamar et al., 2005; Klem, 2009). Currently, rabbit antithymocyte globulin and anti-T lymphocyte globulins are widely used in clinical therapy (Lytton et al., 2007). In this study, we produced anti-rabies neutralizing antibody by using rabbit as a bioreactor through immunization with inactivated rabies virus. And it was characterized by reactivity with rabies antigens, affinity assay, IFA and immunocytochemistry.

In order to prepare neutralizing antibodies which could be better applied to patients for preventing the challenge of rabies virus, especially for prevalent...
Chinese rabies strains, CTN strain of the rabies virus was chosen as the immunogen, as the structure of GP gene is basically stable, and the homology of CTN to street strain is higher than those of aG and PV strains (Ming et al., 2006). Neutralizing antibody titer against rabies virus was certainly detectable by RFFIT (6.7 IU/ml), and comparable to those in human volunteers (6 IU/ml) immunized with the rabies vaccine (Crawford et al., 1980; Chantanakajornfung et al., 1999) or horses immunized with the conventional method (Goel et al., 2003; Redwan et al., 2009).

Several studies confirmed that the glycoprotein (GP) is the important antigen of the rabies virus; it is capable of inducing and binding neutralizing antibodies (VNA) to the virus, which confer immunity against a lethal challenge with the virus (Dietzschold et al., 1990; Mebatsion et al., 1995). In the present study, the western blot (Fig. 3) showed that rabbit antirabies IgGs could bind with glycoprotein (67kD) of rabies virus, suggesting that the rabbit antirabies IgGs might have the ability to neutralize rabies. The neutralizing activity of rabbit anti-rabies analysis in vitro on cultured BSR cell by RFFIT demonstrated that rabbit anti-rabies IgGs were capable of inhibiting rabies virus infection.

Furthermore, in vivo protection studies using the antibodies indicated that treatment of Kunming mice with RRIG resulted in protection that was higher (25%) than that offered by HRIG when mice were challenged with a lethal RV dose. The reason RRIG can provide more protection than HRIG might be that RRIG recognize different epitopes; or RRIG are generally of a higher affinity than HRIG. Unfortunately, RRIG and HRIG could not protect all mice from rabies virus challenge. The reasons for this phenomenon may be that the injection site of virus challenge was in the left forelimb of mice in this study, which extremely adjoins the central nervous system, or the virus would have invaded the nervous system before injected vaccine and RRIG/HRIG.

The results indicate that the current HRIG or ERIG can be replaced by rabbit anti-rabies neutralization antibody which may provide the advantages of specific protective activity, consistency in biological activity, and less immunogenicity. Small animals like rabbit have further advantages over horse of low cost incurred and convenience to farm.

The current results indicate the possibility of obtaining nearly an equivalent amount of RIG, without harnessing a big animal (horse) or human, by rabbit immunization. Further studies are still needed to increase the unit/ml yield product of high efficacy, higher protection rate and to improve the safety profile including the use of pepsin digestion to split the Fc part of the immunoglobulin molecule, which is responsible for inflammatory reactions and complement activation, then removed by a precipitation step. This process has been reported to yield purified F(\(\text{ab}'\))2 fragments (the antigen binding fragment) containing the antigen binding site and the immunoglobulin recognition function (Quiambao et al., 2008).

In conclusion, although further studies are necessary, this kind of rabbit anti-rabies IgGs is safer and more economical drug for PEP, especially for the people living in developing countries where it is difficult to obtain not only HRIG but also ERIG after exposure to life threatening rabies virus.

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