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Isolation and characterization of human neutralizing antibodies to rabies virus derived from a recombinant immune antibody library

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A human immune Fab library was constructed using RNAs from peripheral blood lymphocytes obtained from rabies virus hyperimmune volunteers on phagemid vector. The size of the constructed Fab library was 2×10^7 *Escherichia coli* transformants. After four rounds of panning on whole inactivated rabies virus (PV-11), phage clones displaying rabies virus-specific human Fab were selected. The specificity of soluble Fab antibody fragments, derived from positive phage clones was verified by ELISA. Among 20 specific Fab clones, the genetic sequence of 6 of them (FabRV01, FabRV02, FabRV03, FabRV04, FabRV05, and FabRV06) was analyzed. The variable heavy (VH) and variable light (VL) domains were found to share 90% and 93% homology with sequences encoded by the corresponding human germline genes, respectively. The soluble Fab fragments, expressed in *Escherichia coli* were purified by a single step Nickel-NTA affinity chromatography via a hexa-histidine tag and their binding specificities to rabies virus were confirmed. Three of the Fab antibodies, FabRV01, FabRV02 and FabRV03, showed binding characteristics to rabies virus glycoprotein antigenic site III with affinities in the K_D range 7×10^{-9} to 5×10^{-8} M. The Fab fragments showed dose-dependent neutralization properties for the challenge virus standard (CVS-11).

1. Introduction

Rabies is an endemic, fatal zoonotic disease which is transmitted by the bite of an infected animal, usually from a dog (Cleaveland et al., 2003; De Hoff and Ross, 1981; Jackson, 2003). Rabies is acute, progressive, and incurable viral encephalitis (Dietzschold et al., 2005; Hemachudha et al., 2005). The causative agent is a neurotropic RNA virus in the family of *Rahbdoviridae*, genus Lyssavirus (Coulon et al., 1994). Rabies virus (RABV), is a negative-stranded RNA virus, is a member of the genus *Lyssavirus* within the family *Rhabdoviridae* (Nadin-Davis and Fehlner-Gardiner, 2008). Five structural proteins, including the nucleoprotein (N), phosphoprotein, matrix protein (M), glycoprotein (G), and RNA-dependent RNApolymerase (L), are encoded by the 12-kb viral genome (Wunner et al., 1988). Rabies glycoprotein G (RVG), the only protein exposed on the surface of the viral particle (Langevin et al., 2002), is the mediator of both binding to cellular receptors and entry into host cells, and is a highly immunogenic protein inducing virus-neutralizing antibodies that protect against RABV infection (Cox et al., 1977; Sakamoto et al., 1999; Wiktor et al., 1992). Furthermore, RVG induces cytotoxic T lymphocytes (Macfarlan et al., 1986) and T helper cells (Celis et al., 1988).

The World Health Organization (WHO) estimates human mortality from rabies to be 55,000 deaths per year. However, rabies is preventable: even in case of severe rabies exposure (category III according to WHO guidelines), post-exposure prophylaxis (PEP) consisting of thorough wound cleaning and immediate administration of rabies immune globulin (RIG) together with a full course of rabies vaccination is highly effective (Rupprecht and Gibbons, 2004; Wilde and Hemachudha, 2005; Wilde et al., 2002). More than 10 million people are reported to receive PEP every year throughout the world (Dietzschold et al., 2003; Rupprecht et al., 2008). The administration of RIG as soon as possible after exposure to rabies virus is essential to inhibit viral spread in the interval before sufficient immunity is developed in response to vaccination (Rupprecht et al., 2002). Currently, human (HRIG) and equine (ERIG) immune globulins are used (Carrieri et al., 2006; de Kruif et al., 2007; Goudsmit et al., 2006). These plasma-derived, polyclonal products are obtained from human donors vaccinated against rabies or horses and can only be produced in limited amounts. In addition, the recommended HRIG is expensive, not easily available; suffers from potential drawbacks, such as limited capacity, and batch-to-batch variation, and carries the risk of infections associated with human material (Wilde et al., 2002). ERIG has the disadvantage of an animal origin, and therefore may cause severe hypersensitivity reactions (Satpathy et al., 2005). Therefore, the WHO encourages strongly the development of alternative products

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(WHO consultation on a rabies monoclonal antibody cocktail for rabies post-exposure treatment) (Bakker et al., 2005; Bakker et al., 2008).

Human antibodies constitute the most rapidly growing class of human therapeutics and the second largest class of drugs after vaccines (Brekke and Loset, 2003; Brekke and Sandlie, 2003). Most of the increasing numbers of antibodies entering clinical trials are of human origin (Reichert, 2008; Reichert et al., 2005) and are derived from phage display technology (Hoogenboom, 2005) or from transgenic mice that express human immunoglobulin genes (Lonberg, 2005). However, the best mAbs for clinical applications should be derived from natural human antibodies generated as a result of the *in vivo* immune response for the following three reasons: (i) they are products of the human and not animal repertoire, and (ii) being the results of a human immune response, they should minimize the risks of reactivity against self-antigens. Lastly, (iii) passive immunotherapy with human anti-rabies IgG has been shown to confer immediate protection without the side effects linked to the use of chimeric or humanized mAbs containing animal-derived amino acid sequences. Furthermore, considerable evidences indicate that human antibodies represent a new approach to the development of therapies against bacterial and viral pathogens that causes disease in individuals with impaired immune response and/or for which there are few or no available drugs (Casadevall et al., 2004; Lanzavecchia et al., 2007; Sawyer, 2000; Simmons et al., 2007).

To investigate the Abs produced in humans vaccinated against rabies virus, an immune Fab library was constructed using RNAs extracted from peripheral blood lymphocytes of hyperimmune volunteers. Specific human Fab fragments have been affinity selected from this library on whole inactivated rabies virus. The nucleotide sequences of Fab region in the heavy- and light-chains were analyzed and their corresponding complementarity determining regions (CDRs) were compared. Three Fab clones were shown to recognize different epitopes on rabies glycoprotein antigenic site III and to have the capacity to neutralize rabies virus *in vitro*.

2. Materials and methods

2.1. Human donors

Ten healthy volunteers (four females and six males) working at the Pasteur Institute of Tunis, vaccinated regularly with rabies vaccine (Flury LFP strain, Rabipur®, Chiron Behring Vaccines, Maharashtra, India) were boosted once with the same rabies vaccine at the doses recommended by manufacturer. Five days after the boost, the blood (20 ml) was obtained from each donor and the sera were titrated by the rapid fluorescent focus inhibition test (RFFIT) and the Platelia Rabies ELISA Kit (Biorad, Marnes La Coquette, France). Consent was obtained from the vaccinated donors, all blood samples were anonymised and ethical approval for the study was obtained from the Ethics Committee at the Pasteur Institute of Tunis.

2.2. Vector, E. coli and helper phage

Phage display DNA vector pComb3X (4.8 kb; (Andris-Widhopf et al., 2000)) was used for human Fab library construction and propagation in *E. coli* strain XL1-Blue (Stratagene, La Jolla, USA) that suppresses the amber stop codon between the end of the heavy (H) chain and the phage coat protein III to generate Fab-phage. The pComb3X was also used with non-suppressor strain Top10F′ (Invitrogen, Cergy Pontoise, France) to produce soluble Fab containing both HA-epitope and His6x-purification tags (Andris-Widhopf et al., 2000).

2.3. Titration of rabies antibodies

Titration of antibodies to rabies virus in sera of immunized volunteers was determined by ELISA assay using the Platelia Rabies kit (Biorad, Marnes la Coquette, France), according to the manufacturer's recommendations. Results are expressed in EU/ml (Equivalent Unit per ml of serum) (Anders and Vakil, 2007). Rabies virus neutralizing antibodies were assayed by RFFIT and titers were expressed in international units per milliliter of serum (IU/ml) using a WHO standard as reference (Fitzgerald et al., 1979; Gluck et al., 1987).

2.4. Lymphocyte RNA preparation and human Fab antibody library construction

Human lymphocytes from hypeimmunized donors were separated on a Ficoll-Hypaque gradient (Amersham Biosciences, Saclay, France) and total RNAs were extracted using the QIAamp® RNA Blood Mini Kit (Qiagen, Courtaboeuf, France). cDNA was synthesized using the SuperScript preamplification System (Invitrogen, Cergy Pontoise, Farnce) and one of the constant-region primers CG1z, CK1z, or CL2 (Coronella et al., 2000) and used as template for PCR amplification of the human light and heavy gene fragments. A phage library displaying human Fab antibodies was constructed according to published protocols with minor modifications (Andris-Widhopf et al., 2000; Barbas et al., 1991). The Fab library was constructed by sequential cloning into the phagemid vector pComb3X of the light chain (Vκ–Cκ and Vλ–Cλ) using *Sac*I and *Xba*I followed by Fd fragment (VH-CH1) using *Xho*I and *Spe*I. The Fab library was generated by transforming the resulting recombinant DNA into electrocompetent *E. coli* XL-1 Blue strain, and rescued by infection with the helper phage VCSM13 (10^{11} plaque forming units/ml). Phages were precipitated from the culture supernatant by addition of 20% polyethylene glycol (weight/volume) and 2.5 M NaCl, resuspended in phosphate buffered saline (PBS), pH 7.4 and stored at −20 °C.

2.5. Analysis of Fab V sequences

The Fab genes were amplified by PCR with pFabFor (5′AGAGCGCCCAATACGCAA3′) and pFabBack (5′TAGCCCCCTTATTAGCGTTTGCCA3′), and the nucleotide sequences of heavy- and light-chain genes from chosen clones were determined by an automated DNA sequencer machine (Applied Biosystems 3130, Pasteur Institute of Tunis) using a Taq fluorescent dideoxynucleotide terminator cycle sequencing kit (Applied Biosystems). The sequencing primer, VHPelBseq (5′ACCTATTGCCTACGGCAGCCG3′) and VLOmpseq (5′AAGACAGCTATCGCGATTGCAG3′) were used respectively for the VH and VL gene analyses (Andris-Widhopf et al., 2000; Barbas et al., 1991). Sequences of VL and VH genes were analyzed and compared with human immunoglobulin genes, using the IMGT/V-QUEST and IMGT/JunctionAnalysis software provided (Lefranc et al., 2003).

2.6. Biopanning of human Fab phage library on inactivated rabies virus

Biopanning of the Fab library was carried out on whole inactivated rabies virus, using standard protocols (Andris-Widhopf et al., 2000; Barbas et al., 1991). Maxisorp microtiter plates (Nunc, Roskilde, Denmark) precoated with inactivated rabies virus (PV 11) at 4 °C overnight in PBS, pH 7.4 at the concentration of 1 U/ml, 0.5 U/ml, 0.5 U/ml or 0.25 U/ml for the first, second third and fourth round, respectively. After the fourth round, random phage-Fab