ISSN 0006-2979, Biochemistry (Moscow), 2018, Vol. 83, No. 1, pp. 1-12. © Pleiades Publishing, Ltd., 2018.
Original Russian Text © E. N. Ilina, M. V. Larina, T. K. Aliev, D. A. Dolgikh, M. P. Kirpichnikov, 2018, published in Biokhimiya, 2018, Vol. 83, No. 1, pp. 3-18.

REVIEW

Recombinant Monoclonal Antibodies for Rabies Post-exposure Prophylaxis

E. N. Ilina[1]*, M. V. Larina[2], T. K. Aliev[3], D. A. Dolgikh[1,2], and M. P. Kirpichnikov[1,2]

[1]*Lomonosov Moscow State University, Faculty of Biology, 119991 Moscow, Russia; E-mail: ekaterina.iljina.7@yandex.ru*
[2]*Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117997 Moscow, Russia*
[3]*Lomonosov Moscow State University, Faculty of Chemistry, 119991 Moscow, Russia*

Received July 6, 2017
Revision received September 11, 2017

Abstract—Rabies virus is a prototypical neurotropic virus that causes one of the most dangerous zoonotic diseases in humans. Humanized or fully human monoclonal antibodies (mAb) that neutralize rabies virus would be the basis for powerful post-exposure prophylaxis of rabies in humans, having several significant benefits in comparison with human or equine rabies polyclonal immunoglobulins. The most advanced antibodies should broadly neutralize natural rabies virus isolates, bind with conserved antigenic determinants of the rabies virus glycoprotein, and show high neutralizing potency in assays *in vivo*. The antibodies should recognize nonoverlapping epitopes if they are used in combination. This review focuses on basic requirements for anti-rabies therapeutic antibodies. The urgency in the search for novel rabies post-exposure prophylaxis and methods of development of anti-rabies human mAb cocktail are discussed. The rabies virus structure and pathways of its penetration into the nervous system are also briefly described.

DOI: 10.1134/S0006297918010017

Keywords: human monoclonal antibodies, rabies virus, glycoprotein G, antigenic site, post-exposure prophylaxis, rabies immunoglobulin

Rabies is one of the deadliest zoonotic infectious diseases because it is fatal to humans in most cases. Even now, in the XXI century, rabies is one of the most important human health hazards. The disease occurs on all continents except for Antarctic, but most cases of the infection occur in Africa and Asia, where several tens of thousands of fatal cases are recorded annually. However, data on fatal cases of infection with rabies virus (RABV) assessed as 60,000 humans per year are incomplete due to the absence of appropriate epidemiological control and laboratory infrastructure [1]. Nearly all cases of RABV infection in humans (most often children under the age of 15) are related to bites from infected dogs.

Rabies remains a serious problem for healthcare throughout the world, and especially in developing countries. Fatal cases from RABV infection can be prevented using modern vaccines, both before and after the infection. However, it is recommended to use rabies immunoglobulin (RIG) in addition to vaccines in most cases of infection in humans to enhance the efficiency of anti-rabies prophylaxis. In addition to equine rabies immunoglobulin (ERIG), donor human rabies immunoglobulin (HRIG) is also used for passive immunization because it is considered safer than ERIG. Extensive use of HRIG in developing countries is impeded by its high cost and an insufficient number of donors. Thus, substitution of HRIG by rabies immunoglobulin of nonanimal origin, which is at least comparable in efficiency and safety, is still a significant problem whose solution will increase the availability of anti-rabies drugs. Human antibodies, or humanized monoclonal antibodies (mAb), can be the best alternative in rabies prophylaxis in humans. In contrast to polyclonal RIG bound to different, including neutralizing, epitopes of RABV, some mAb interact with only one viral epitope. The potential problem of using mAb can be a change in or even absence of the target epitope in some viral strains, which can lead to a decrease in cross-specificity of the

Abbreviations: AS, antigenic site; CHO, Chinese hamster ovary tumor cells; ERIG, equine rabies immunoglobulin; (H)RIG, (human) rabies immunoglobulin; IFN, interferon; mAb, monoclonal antibodies; nACR, nicotine acetylcholine receptor (n-cholinoreceptor); NBs, Negri bodies; NCAM, neuronal cell adhesion molecule; NGFR or p75NTR, nerve growth factor (p75 neutrophin receptor); PEP, post-exposure prophylaxis; RABV, rabies virus; RIG, rabies immunoglobulin; STAT, transcription factor; TLRs, toll-like receptors.

* To whom correspondence should be addressed.

mAb. However, even if the epitope is highly conservative, when using mAb the viral variants selected because of selective pressure may have point mutations in the region of the epitope, which impairs the binding of these variants by a single therapeutic antibody.

Thus, it is unsafe to use a single mAb for the prevention and treatment of infections caused by viruses occurring in the environment, because different strains and variants and/or those characterized by high levels of replication after infecting a host. This problem can be solved using a cocktail of several noncompeting mAbs. The mAb components of the cocktail must not be inferior to HRIG in efficiency and must meet certain requirements. First, mAbs must have high activity to provide considerable neutralization of RABV with limited doses of the antibody. Second, the antibody cocktail must have a neutralizing effect on a broad range of natural RABV variants. Third, the mAb components of the cocktail must bind to separate nonoverlapping epitopes, preventing some virus mutants from escaping neutralization. Finally, in addition to efficiency *in vitro*, the mAb cocktail must protect from the development of fatal infection caused by RABV in combination with anti-rabies vaccine in tests with relevant models *in vivo*.

The availability of a mAb cocktail that would be no less effective than the anti-rabies polyclonal globulins already used for rabies prophylaxis can lead to wider application of passive immunization in developing countries and, consequently, to a decrease in the number of fatal cases of RABV infection in humans.

MOLECULAR BASES OF RABIES PATHOGENESIS

Rabies virus. The rabies disease in humans and animals is caused by (−)ssRNA RABV from the genus *Lyssavirus*, family Rhabdoviridae, order Mononegavirales, group V of the Baltimore classification [2]. There are also cases of fatal encephalitis in humans caused by other members of the *Lyssavirus* genus [3], which are recorded much less frequently. RABV is the first species that has been studied and characterized among 14 identified viral species of the *Lyssavirus* genus [4].

Lyssaviruses are bullet- or rod-shaped and have a genome of nonsegmented negative single-stranded RNA contained within a capsid coated with a host-cell bilipid membrane. The genomic RNA (12 kb) encodes five proteins: N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein), and L (RNA-dependent RNA polymerase). The N, P, and L proteins, together with the RNA, form a ribonucleoprotein complex surrounded by a lipid envelope associated with the M- and G-proteins. The G-protein forms trimers and is the major surface antigen to which the neutralizing antibodies are bound. The viral virion includes 500-1600 G-protein molecules, making up one-fourth of the protein.

Rabies pathogenesis. The binding of lyssaviruses with cells involves a viral glycoprotein that interacts with receptors providing virus entry into the cell via clathrin-mediated endocytosis [5]. The RABV glycoprotein is a homotrimer protruding as spikes from the virion surface. The G-protein, having the key role in viral infection, participates in several stages of the life cycle of the virus: it allows viral particles to be fixed on the host cell surface and thereby determines cellular tropism [6]. After binding to the receptor, the virus enters the cell via endocytosis. The acidic medium of endosomes induces conformational changes in the G-protein molecules; as a result, the viral envelope is fused with the cell membrane, and the viral nucleocapsid is released into the cytoplasm. After the cycle of reproduction in the cell, the virus buds off, also with involvement of the G-protein. In addition, the glycoprotein plays a key role in the induction of host immune response, induces the synthesis of specific neutralizing antibodies [7], and stimulates the cytotoxic activity of T-lymphocytes [8].

The mature glycoprotein molecule consists of 505 a.a. residues. The G-protein molecule has three domains: an ectodomain (a.a. 1-439), a transmembrane domain (a.a. 440-461), and a cytoplasmic domain (a.a. 462-505). Among the three potential *N*-glycosylation sites (Asn37, Asn247, Asn319) predicted for this glycoprotein, only one or two sites are glycosylated, the first with a minimum level of glycosylation [9]. Another posttranslational modification of glycoprotein G is the attachment of fatty acid residues, palmitoylation, at Cys460 [10].

The antigenic structure of the RABV glycoprotein was determined using mAb panels. (The numeration of amino acid residues is indicated for the mature protein, after signal peptide removal.) Antigenic site (AS) I includes both conformational and linear epitopes and is represented by a.a. 226-231 [11]. AS II is a conformational epitope including a.a. 34-42 (IIb) and a.a. 198-200 (IIa) [12]. AS III is also a conformational epitope containing a.a. 330-338. AS IV includes a single amino acid residue in position 251. The minor site *a*, also referred to as G1, is localized in position a.a. 342-343. AS G5 is a linear epitope including a.a. 261-264, which also comprises antigenic site VI previously described as a site with a single amino acid residue in position 264 [13]. The positions of antigenic sites of the RABV glycoprotein are shown in Fig. 1.

In vitro, lyssaviruses can infect a wide range of cell lines, though the mechanisms of their penetration into different cells are unclear. It is supposed that carbohydrate fragments, phospholipids, and sialylated gangliosides can act as potential sites of virus binding and entry into cells [14].

RABV is a pathogen that is well adapted to the mammalian nervous system and affects mainly neurons. As a result of a bite and muscle infection, viral particles from the saliva of infected animals penetrate the nervous system of the host via sensory nerves or neuromuscular junc-

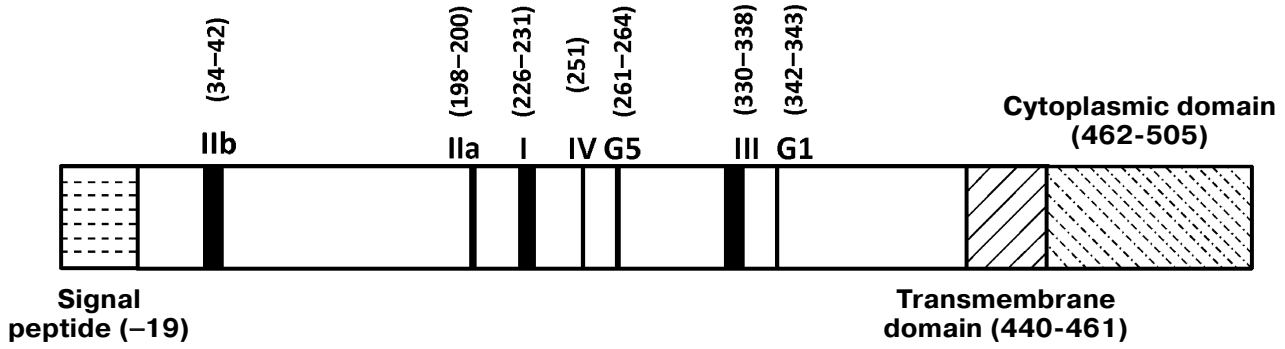

Fig. 1. Antigenic sites of RABV glycoprotein. Numerals denote amino acid residue numbers. The schematic drawing shows amino acid residue numeration and relative positions of antigenic sites (I, IIa, IIb, III, IV, G1, and G5) within the extracellular domain G. The numeration corresponds to the mature peptide.

tions where motor axons divide into several terminals and contact muscle fibers. The virus moves from peripheral nerve endings to the spinal cord, from which it then penetrates the brain. Only after active amplification in the brain is the virus transferred to lymph or saliva glands for subsequent spreading. Mature virions enter saliva and are transmitted to other animals through bites.

The virus begins to multiply during the incubation period lasting from several weeks to several years, 1-2 months on average. During the greater part of the incubation period, viral particles remain in muscles or begin to slowly replicate while staying at the bite site. The host organism does not develop an immune response to local infection. Dendritic cells playing the key role in the induction of both innate and adaptive immune response are not sufficiently activated by the virus. The long-term incubation period is probably determined by the low titer of viral particles in tissues, as well as by the presence of endogenous microRNA that slow viral replication in muscles. It is supposed that microRNA mir-133 specific for muscle cells are bound to the RNA encoding RABV nucleoprotein and glycoprotein, which prevents the expression of viral proteins to a considerable extent, as confirmed in experiments with Neuro-2a cells transformed by rhabdovirus [15]. However, experiments with mice and primates have shown that RABV at high concentration is able to infect motor endplates without prereplication in muscles [16]. Moreover, when entering directly into nervous tissue at high titer, the virus can infect motor neurons without prereplication [17]. This discovery accounts for the extremely short incubation period in RABV-infected humans after penetrating nerve injury.

The literature data show that RABV attacks motor neurons via neuromuscular junctions [18]. It uses as cellular receptors membrane molecules and mechanisms involved in the transport of growth factors, hormones, nutrients, and other vitally important molecules into cells. Three types of RABV receptors have been detected, with which it is specifically bound or which facilitate its penetration into cells. These are the nicotine acetylcholine receptor (n-cholinoreceptor, nACR), the neural cell adhesion molecule (NCAM) and the nerve growth factor receptor often referred to as p75 neutrophin receptor (NGFR or p75NTR) (Fig. 2).

At the neuromuscular junction, free viral particles are bound to nACR localized in the synaptic cleft on the postsynaptic membrane surface. At the same time, RABV concentration on the membrane surface increases, thereby enhancing the probability of virus particle capture on nerve endings [18]. There are several types of nACR in the nervous system, but only one form containing the $\alpha 1$-gene product is expressed in muscles, on the postsynaptic membrane. The main G-protein-binding site is the nACR α-subunit fragment between a.a. 173 and 204 [19].

The subsequent movement of RABV to the presynaptic membrane is associated with NCAM molecules concentrated in synapses on the neuronal membrane and at neuromuscular junctions. NCAM molecules are involved in the movement and turnover of synaptic vesicles. The presence of these molecules, together with gangliosides, within lipid rafts results in the binding of the glycoprotein to NCAM, detachment of viral particles from nACR, and the movement of virions towards nerve endings.

RABV particles penetrate cells while being inside vesicles; their envelope can fuse with the vesicle membrane and, as a result, a nucleocapsid is released. In an alternative pathway of spread, viral particles stay in the vesicles and are transported to the soma of neurons, where their replication is possible. The RABV glycoprotein can bind to the receptors of nerve growth factor p75NTR [20]; as a result, viral particles move along the axon by the mechanism of retrograde transport via the spinal cord to the brain. Before this stage, usually no clinical signs of the infection are observed.

Other cell membrane components are also involved in viral transport into cells. Phospholipids, glycolipids, sialic acids, and carbohydrates are supposed to participate in RABV entry into a cell. The available data demonstrate

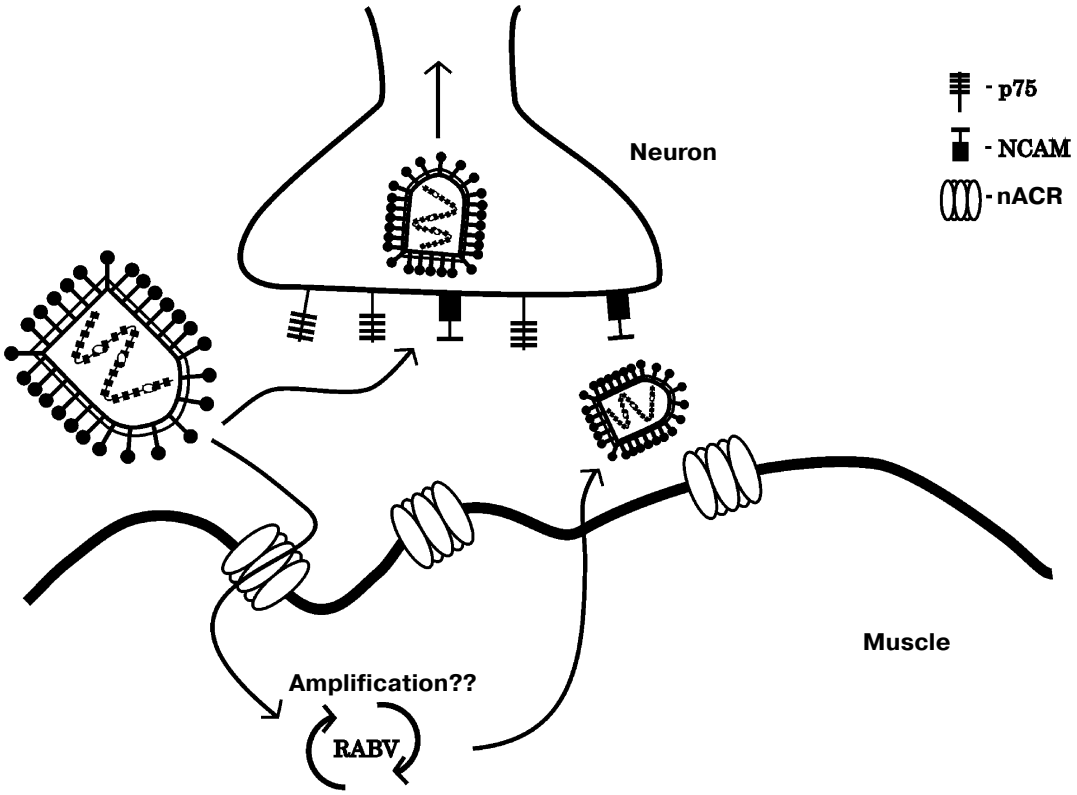

Fig. 2. Mechanisms of RABV penetration into neurons.

that the highly sialylated gangliosides, which are the main component of the presynaptic membrane, also mediate viral entry into cells [21]. Nevertheless, it has not yet been proved that RABV is bound directly to gangliosides.

Escape from immune recognition. The human and other mammalian immune systems are unable to cope with RABV without active therapy. The virus has developed several ways of escaping recognition by both the innate and the adaptive immunity. The basic mechanism of forming an intracellular response to viral infection is implemented via the two classes of innate sensors – helicases RIG-1 (DDX58) and MDA5 (IFIHI) [22], as well as transmembrane Toll-like receptors 3 and 7/8 (TLRs) [23]. TLR3 recognizes exogenous or endogenous double- or single-stranded RNA molecules while being located on the outer plasma membrane of the cells and on the endosomal membrane. Thus, the infected neurons and neuroglial cells can induce immune response using the antiviral mechanisms of innate immunity, namely, type I (α and β) interferons (IFN), as well as antiinflammatory cytokines and chemokines. Interferons, by activating interferon-stimulated genes, via a complex cascade of events finally lead to degradation of both viral and cellular RNAs. This can cause cell death, which is supplementary protection for the entire organism. However, it has been shown that the virus uses TLR3 for its own purposes, suppressing interferon synthesis. The replication and transcription of the virus begin after its entry into the neuronal body by endocytosis. As a result, inclusions are formed in the cytoplasm, so-called Negri bodies (NBs), previously considered as sites of accumulation of excessive amounts of viral proteins [24]. However, more recent studies have shown that NBs are the sites of virus transcription and replication [24]. At the same time, TLR3 is the major protein involved in formation of NBs, in addition to viral proteins N, P, and viral RNA contained in these bodies [25]. The accumulation of TLR3 in viral aggregates in the cytoplasm decreases the activation of innate immunity sensors and interferon production. At the same time, the RABV phosphoprotein blocks type I IFN production by inhibiting IFN-α and IFN-β gene transcription, thereby preventing the phosphorylation of TBK1 and IKK-i by the IRF3 and IRF7 kinases. The STAT (STAT 1 and STAT 2) signaling pathway, by which type I IFNs implement their antiviral activity, is also inhibited [26]. Thus, the inflammatory process in the central nervous system invaded by the viral particles is largely suppressed, allowing the virus to spread in the human organism more rapidly.

The suppression of the adaptive immune response is associated with the apoptosis of T cells and monocytes that have penetrated through the blood–brain barrier to fight the virus directly in nervous tissue. RABV uses for its purposes the ability of T cells to control their activation

via inhibitory stimulation or apoptosis, which normally regulates their tolerance and weakens the immune response. The neurons infected by the virus express on their surface an increased number of inhibitory molecules, which interact with the respective T cell receptors and thereby inhibit their proliferation and cytokine production, or induce the apoptosis of this subpopulation. With the purpose of suppressing the effector immune responses of the host, RABV uses the PD-1/B7-H1, CD8/HLA-G, and Fas/FasL-mediated pathways of inducing the apoptosis of T cells [27].

The escape from recognition by the innate immunity system and the suppression of the adaptive immune response can be considered as a successful adaptation of RABV to a host organism.

RABIES PROPHYLAXIS IN HUMANS: BEFORE AND AFTER INFECTION

Pre-exposure or post-exposure (PEP) prophylaxis is currently the only way to prevent the development of rabies. Pre-exposure prophylaxis includes prevaccination, which is necessary for enhanced infection hazard. PEP is performed immediately after the contact with animals suspected of having rabies. This method of prophylaxis is currently achieved through the application of polyclonal sera and vaccines that must be combined for effective suppression of development and the elimination of viral particles in an organism.

Anti-rabies vaccines for humans. Vaccination is aimed at inducing the formation of specific virus-neutralizing antibodies. Being a highly effective strategy in the prevention of disease development, vaccination is performed in the framework of both pre-exposure prophylaxis and PEP. The advantages of prophylactic immunization are as follows: the vaccine dose necessary for PEP is lower in case of real infection, and often passive immunotherapy with RIG is not required.

The first anti-rabies vaccines were based on unpurified preparations of animal nervous tissues; they were weakly immunogenic, so several doses had to be administered to induce immune responses. In the 1940s, they were replaced by more immunogenic and safer cell-culture-derived vaccines (CCVs) [28]. Despite official recommendations to discontinue the application of nerve-tissue vaccines, they are currently used in some countries.

The large-scale production of inactivated CCVs in 1960 made it possible to limit the spread of RABV [28]. The anti-rabies vaccine became widely available for the first time for the prophylaxis of human disease before a bite, not only after infection.

In the past two decades, the development of new-generation vaccines for both humans and other animals has continued. These studies have resulted in the production of recombinant anti-rabies vaccines containing a biotechnologically synthesized antigen. The recombinant antigen for the vaccine is obtained by cloning the RABV (strain ERA) glycoprotein gene in bacterial or viral vectors [29], followed by its expression in the respective heterologous systems. It should be noted that the recombinant antigen-based vaccines are less hazardous for humans as they do not contain viral particles. Recently presented prototypes of plant-based recombinant vaccines were obtained using plant virus vectors (such as tobacco mosaic virus (TMV)) [30]. The plant-based production systems are inexpensive, safe, and easy-to-use. In addition, experimental DNA vaccines are being developed: the gene encoding an immunogenic protein is incorporated in a vector that provides its expression but is incapable of independent replication in a host organism. The products of expression of these genes can cause immunity formation, remaining in an organism for up to 3-4 weeks [31]. Such vaccines have not yet been extensively used in clinical practice as their application requires various investigations to confirm their safety and efficiency. It should be noted that such great progress in this trend of vaccine development will probably soon result in creation of new efficient and safe vaccines recommended for application in human and veterinary medicine.

Post-exposure prophylaxis. Rabies is one of the few infectious diseases whose development can be prevented by PEP after viral entry into an organism. The key issue of PEP efficiency is the period of time when these measures are taken.

Each year, more than 20 million people all over the world have PEP preventing hundreds of thousands of deaths. PEP includes the immediate washing and disinfection of all bite and scratch wounds, the administration of RIG, and the complete course of vaccination. When PEP is performed in accordance with the regulations, RABV can be removed from an organism before the beginning of reproduction of viral particles in the central nervous system tissues.

Urgent PEP is a highly effective approach combining both active and passive immunizations. The goal of the passive immunization with RIG is to form short-term immunity preventing virus replication in the period of time necessary for the development of immune response and the appearance of neutralizing antibodies in the human organism after the vaccination. The combination of anti-rabies vaccine and RIG is nearly 100% efficient in preventing the disease development. Unfortunately, the application of the vaccine or RIG after the appearance of rabies symptoms is ineffective, and fatal outcome is almost inevitable [32].

Polyclonal anti-rabies immunoglobulins (RIG). RIG has been used for rabies PEP in humans since the middle of the XX century. The advantages of polyclonal immunoglobulins are their high efficiency, broad-range virus-neutralizing activity, polyspecificity, and, when using HRIG, effector functions. The most important fea-

ture of RIG is the ability to interact with several antigenic epitopes (polyspecificity), which prevents the selection of mutant virus variants escaping from the neutralizing effect of the antibodies.

The RIG production process usually includes the purification of class G immunoglobulins (IgG) from plasma of immune humans (HRIG) or other animals (usually equine, ERIG). HRIG and ERIG are currently used for rabies PEP in humans. ERIG, which is used mainly in developing countries, is rather highly immunogenic for humans. The administration of ERIG induces the formation of anti-equine antibodies, resulting in the rapid release of ERIG from the human organism. The short period of equine immunoglobulin circulation requires the use of high doses, which may lead to the development of type III hypersensitivity reaction, sometimes resulting in fatal outcome. Because of adverse responses, physicians are often reluctant to use ERIG, which contradicts the standards of complete PEP and can lead to ineffective rabies prophylaxis with fatal outcome. The side effects of equine immunoglobulin therapy are associated mainly with the Fc-domain of antibodies and, consequently, ERIG lacking this region, i.e. $F(ab')_2$ obtained by pepsinolysis of IgG, is a less hazardous drug that is currently used together with the full-size immunoglobulins [33]. In addition to high immunogenicity, ERIG also poses certain infection hazards to people, and hence it requires high-quality purification.

The search for an alternative to serum products of animal origin has resulted in the production of HRIG that is a functional analog of ERIG. The modern HRIG is safe, nonimmunogenic, and well tolerated by people. There are very few known cases of anaphylaxis or serum sickness. However, since HRIG is isolated from human plasma, there is a potential hazard of transmission of blood-carried infectious agents, which can be reduced by detergent or thermal treatment of the serum. Control system that includes the screening and testing of donors, the observance of GMP principles in production, and the specific process of virus inactivation considerably lowers risk. Such processes are expensive and usually not available in developing countries, where 90% of RABV infection cases are recorded [34, 35].

Polyclonal RIG, regardless of their origin, generally has certain disadvantages, including the necessity of using donors and their immunization; ethical problems; lower specific activity (i.e. greater amounts of protein are required, which can lead to side effects); batch-to-batch variation; potential risk of transmission of infectious agents. On the grounds of medical safety and enhanced availability of the drug in developing countries, it seems necessary to replace HRIG and ERIG by alternative products, as follows also from WHO recommendations [36].

Monoclonal anti-RABV antibodies. The use of monoclonal antibodies in therapy (inter alia, for viral diseases) presupposes several advantages compared to their polyclonal analogs: mAb have higher specific activity, because all immunoglobulin molecules contained in the preparation are specific against the selected target. Hence, protein amounts can be lower, and the volume of injection can also be decreased, avoiding the development of unfavorable responses. Standardized production from cell producers provides batch-to-batch homogeneity of mAb preparations. There are also some disadvantages of mAb application, which, however, have no appreciable influence on the prospects of using antibodies in therapy for infectious diseases. The of production of mAbs is rather expensive. At the same time, despite the high specificity of mAbs, pathogenic organisms capable of rapid antigenic variation create significant obstacles to the development of broad-spectrum neutralizing antibodies. Since a mAb is bound to a single epitope, variants of pathogenic viruses escaping neutralization due to mutations or microevolutionary processes can be selected in nature.

An mAb-based immunoglobulin preparation for urgent PEP of rabies must meet some requirements. First, the antibodies must have neutralizing activity against RABV variants that are most widespread in the region where these therapeutic antibodies are used. Second, the preparation must contain at least two different antibodies bound to nonoverlapping and noncompeting epitopes. Thus, viruses carrying the mutations that allow them to avoid the binding with one of the antibodies can be neutralized by another one [37]. Third, the antibodies must be nonimmunogenic for humans. Fourth, the preparation must be comparable in efficiency with the existing HRIG preparations [37]. In addition, the production cost of anti-rabies antibodies must be acceptable to make them available for rabies prophylaxis in all patients needing PEP, including those in developing countries where the number of fatal cases is the highest.

Detailed description of full-size antibodies proposed as candidates to replace RIG in the framework of rabies PEP in humans is given in the following sections of this review.

BASIC CHARACTERISTICS AND METHODS OF PRODUCTION OF ANTI-RABV mAbs

Antigenic specificity of RABV mAbs. Glycoprotein G is the only RABV antigen that induces the formation of antibodies neutralizing the virus [7]. Mechanisms of neutralization by antibodies have been proposed for different viruses. The simplest one of presumptive neutralization mechanisms is the prevention of virus attachment to a cell due to the blocking of all glycoprotein molecules of the virion. Another potential mechanism is based on the ability of antibodies to cause conformational changes in the G-protein resulting in the loss of ability of the virion to be bound to cellular receptors, which is necessary for virus

entry into a cell and subsequent replication. In addition, virus-neutralizing antibodies may exert their protective effect by removing the pathogen via complement-mediated lysis of virus-infected cells and antibody-dependent cytotoxicity.

Thus far, the immunochemical properties have not been sufficiently studied and characterized for all described mAbs against the RABV glycoprotein. This review is devoted to the antibodies described in the literature for which the neutralizing activity against RABV has been confirmed.

Glycoprotein-binding sites (I, IIa, IIb, III, IV, G5, VI, and the minor site *a*) have been established for most RABV antibodies. The neutralizing antibodies bound to five of eight known AS of the RABV glycoprotein are now known. The mAb candidates for clinical studies that are bound to the minor site *a*, otherwise referred to as G1 (a.a. 342-343), as well as to AS IV (a.a. 251) and AS VI (a.a. 264) have not been described in the literature.

Human antibody CR57 (Crucell) [38], chimeric antibody 62-71-3 [39], and human antibody RVC-20 [40] interact with AS I (a.a. 226-231). Chimeric antibody E559 (AS IIb, a.a. 34-42) [41] and human antibody No. 254 (AS IIa, a.a. 198-200) [42] are bound to AS II. Several human antibodies have been obtained for AS III (a.a. 330-338): CR4098 (Crucell) [43], RABV1 [44], and RVC-58 [40]. Site IV, also known as G5 (a.a. 261-264), interacts with human antibody AR16 [45]. There is one more human antibody, 4D4, that binds to a supposed new epitope of the glycoprotein located between antigenic sites I and VI and including the amino acid residue in position 242 [42].

The antigenic characteristics of the glycoprotein are studied in RABV mutants resistant to neutralization by some mAbs, i.e. by assessing the frequency of substitutions for a particular amino acid residue comprising the antigenic site, and well as the effects of substitutions on the neutralizing activity of the mAb that has manifested this ability previously. Thus, most of the obtained neutralizing mAbs against RABV are bound to AS II or III [13].

The major AS of the G-protein, which is recognized by 70% of the promising mAbs, is AS II. The second most important site is AS III, interacting with ~20% of antibodies against the RABV glycoprotein [46, 47]. In addition, we should mention AS I and the minor site *a*; all other sites are considered as secondary. The importance of various ASs for effective neutralization of a broad range of RABV variants by therapeutic antibodies is associated with their conservativeness. Sites I, II, and III can be considered as most conservative. Each AS is characterized by a different level of amino acid residue variability. In AS I, the amino acid position 231 is most variable; for site II, the maximum variability has been mentioned for a.a. 36, 37, and 199; site III also contains several variable positions – a.a. 332, 333, 336, 338 [40, 48]. Thus, antibodies are preferable for therapeutic application if their specific binding is the least affected by these variable amino acid residues.

A universal mAb that could neutralize all the known RABV variants still seems to be an unattainable goal. The absence of such antibody is accounted for by the great diversity of strains of this virus worldwide, which differ from each other in point mutations localized, inter alia, in antigenic sites, which results in great diversity of epitopes. Thus, the application of mAbs in PEP should be based on a combination of different antibodies, making it possible to neutralize most or all known strains.

Methods of obtaining therapeutic mAbs against RABV. In 1975, owing to the work by Kohler and Milstein [49], the hybridoma technology of mAb production was initiated and the idea of targeted therapy began to take shape. The hybridoma technology made it possible to obtain, in response to immunization by the antigen, not a set of various immunoglobulin molecules, but an antibody targeted against one specific antigen (monoclonal antibody).

Unfortunately, the first generation of mAbs obtained by immunization of mice had the same major disadvantage as ERIG – high rate of immunogenicity in humans. Other limitations, such as instability of some mouse hybridomas, rapid mAb excretion from the organism, and inability of the Fc fragment of animal mAbs to adequately activate protective immunity functions in the human organism due to species differences casted doubt on their universality and safe use in medicine.

The most obvious strategy for obtaining mAbs exhibiting the lowest immunogenicity but at the same time maintaining the necessary antigen-binding properties was chimerization and then humanization of animal mAbs. Chimeric antibodies, with more than 65% of the human immunoglobulin sequence in their structure, retain only the variable domains of the antibodies obtained during immunization of animals. This approach was used to obtain chimeric antibodies 62-71-3 and E559 expressed in tobacco leaves, which are appropriate candidates for a new cocktail of antibodies [39]. In 1986, a humanized monoclonal antibody was obtained assuming that the high percentage of the human immunoglobulin sequence can provide the maximum therapeutic effect and lower immunogenicity. The humanized mAb consists of 95% of the human immunoglobulin, and only the antigen-binding regions (CDRs) were taken from the mouse antibody. An example of humanized anti-RABV antibody is the $(Fab)_2$ fragment of antibody 1C5 expressed in yeast cells [50]. The hybridomas producing anti-RABV mAb were obtained by immunization of mice with RABV strain Vnukovo-32. The panel of antibodies secreted by the hybridomas was tested for the presence of interaction with strains isolated in Russia, Africa, Central Europe, Ukraine, and USA. As a result, the mouse antibody 1C5, which demonstrated neutralizing activity against all the tested RABV strains, was taken for further humanization.

In experiments *in vivo*, the 1C5 antibody showed 100% efficiency of rabies prevention in mice infected by superlethal doses of RABV, while the commercial equine immunoglobulin "Anti-rabies immunoglobulin from equine serum" (BIOLEK CJSC, Ukraine) prevented the death of only 16% of the mice.

The human mAbs are the least immunogenic molecules and, therefore, many modern methods are aimed at obtaining them. Hence, the widely used methods are screening of mini-antibodies (scFv) or Fab fragments of human antibodies of required specificity from combinatorial libraries using a phage, ribosomal and yeast display, followed by construction of full-size human immunoglobulins based on selected fragments. The exposure of peptides and proteins on the filamentous phage surface – the phage display technique – provides the possibility of selecting proteins (including antibodies) with desired properties from a vast set of variants. This technique was used to obtain anti-rabies antibodies CR4098 and AR16. In both cases, antibody libraries were constructed using B-lymphocytes isolated from the peripheral blood of vaccinated donors. The principle underlying this method is the direct relationship between the phenotype of the protein localized on the surface of the phage particle and the respective genotype. Phage display allows the exposure of vast libraries (10^8-10^{11} variants) of peptides and proteins on the surface of a filamentous phage, which are eventually selected towards high affinity and specific binding with nearly all targets. The method is highly productive but has a drawback consisting in the selection of artificial repertoires of antibodies (in the case of a mutant library) that may be immunogenic to humans. Two independent libraries ($1.2 \cdot 10^7$ and $9.3 \cdot 10^6$ clones, respectively) were constructed using the genes of B-lymphocytes from four immune donors with the view to obtain a clone, which subsequently became the prototype of antibody CR4098. The sequencing of random clones from the constructed libraries and their alignment with the nucleotide sequences of germ lines showed the coverage of a great repertoire of donor antibodies. The antibodies were selected using inactivated virus and purified RABV glycoprotein. Considering the conditions of selection of RABV-specific antibodies, there were altogether 40 rounds of biopanning. The antibody AR16 was isolated from the analogous library of $6 \cdot 10^7$ clones from four rounds with the use of synthetic peptide G5-24 comprising AS G5. Peripheral B-lymphocytes were taken from three donors immunized with the Verorab vaccine (Aventis Pasteur, France) containing the inactivated strain Wistar rabies PM/WI 38 1503-3M.

There are some more approaches based on work with human B cells, more exactly, methods of their immortalization. Here it is possible to use human immune B-lymphocytes isolated from peripheral blood (B-cell cloning) or human B-lymphocytes immunized *in vitro*. In both cases, B cells are immortalized by transformation with the Epstein–Barr virus or by fusion with a myeloma partner. The difference consists in the methods of B-lymphocyte activation. In essence, B-cell cloning is the selection of B-lymphocytes that are the most promising regarding their ability to interact with the antigen from a vast pool of clones isolated directly from the peripheral blood of a vaccinated donor. However, immunization *in vitro* implies the activation of B-lymphocytes directly in a test tube, where the conditions for B-cell maturation are created. Human antibodies RVC-20, RVC-58, No. 254 and 4D4 were obtained from transformation of B-lymphocytes by the Epstein–Barr virus, while antibody CR57 was isolated from a heterohybridoma. Both working groups used immune B-lymphocytes from vaccinated donors.

Among the currently known candidate antibodies for RIG substitution in the framework of urgent prophylaxis of rabies, we should first mention the CL184 cocktail consisting of two virus-neutralizing human antibodies that was obtained by the Crucell company. The history of CL184 cocktail development can be divided into two steps. The first antibody CR57, binding to AS I of the glycoprotein, was obtained from B cells of a donor vaccinated against RABV. The resulting B cells were immortalized by creating a heterohybridoma. The CR57 mAb epitope was determined by enzyme immunoassay with a set of peptide components of the glycoprotein as an antigen and a panel of mutants resistant to neutralization. Detailed analysis of the binding site for CR57 mAb showed the minimal region of the glycoprotein including a.a. 226-231 (KLCGVL), among which 4 a.a. (K-CGV) were critical for mAb binding. Analysis of a database containing glycoprotein sequences of a large group of natural RABV isolates showed that three of 229 variants (~1%) carry the mutations that may lead to impaired interaction with CR57 mAb. These findings indicate the high degree of conservatism of the CR57 mAb epitope, which might be associated with the involvement of the central cysteine residue in the folding of the viral glycoprotein molecule [11]. These data were confirmed experimentally: a study of neutralizing activity in 26 street RABV strains showed that the CR57 mAb neutralizes 24 of them *in vitro*. In view of the discovery of neutralization escape mutants of the virus, a partner antibody was sought to be combined with the previously obtained CR57 mAb. The second antibody in the cocktail, CR4098, is bound to AS III of the glycoprotein. This mAb was obtained by phage display technology. The antibodies from the library repertoire were selected using different antigen forms: inactivated virus, native glycoprotein purified from viral particles, and the glycoprotein expressed on the cell surface. The study of the neutralizing activity of antibodies isolated by several rounds of selection revealed 39 unique virus-neutralizing antibodies. An antibody not competing for glycoprotein binding to CR57 mAb, which was selected from among them, neutralized the maximum number of studied viral strains, including the variants escaping neutralization by

the CR57 antibody. CR4098 is a supplement to CR57 and, thus, the combination of these mAbs neutralizes 26 out of 26 tested RABV strains. Later, the efficiency of CL184 was confirmed in animals. The CL184 preparation expressed in PER.C6 cells underwent stages I and II of clinical trials [51-53]; stage III is expected to start soon.

Further improvement of the methods for obtaining human antibodies against the RABV glycoprotein (including neutralizing ones) contributes to the development of representative mAb panels, which makes it possible to select the most promising variants for clinical application. For example, De Benedictis et al. [40] isolated from the blood of four vaccinated donors B-memory cells producing class G immunoglobulins against RABV. The subsequent immortalization of the cells by the Epstein–Barr virus, cloning, and micro-quantitative analysis of supernatants for the ability to neutralize the CVS-11 pseudotype of RABV resulted in the selection of 500 human mAbs, with 21 of them showing the maximum neutralizing activity. The final stage included experiments for the study of the range of neutralizing activity of 21 mAbs against 13 different species of lyssaviruses (22 pseudotypes and 15 live viruses). As a result, it was shown that RVC-20 (bound to AS I) and RVC-58 (bound to AS III) mAbs neutralized all isolates used in the test with high activity.

The development of transgenic mouse lines capable of producing human antibodies includes the following two objectives: knockout of the mouse immunoglobulin genes and the incorporation of human immunoglobulin genes into the mouse genome. Then the given individual is crossbred with a mouse lacking its own immunoglobulin genes. Transgenic mouse production is a very difficult and expensive process, and even the possibility of purchasing ready mice does not promote the spread of this method for obtaining human mAbs. An example of successful work with transgenic mice (Bristol-Myers Squibb, USA) immunized with human vaccines RabAvert® (Novartis, Switzerland) or Imovax® (Sanofi Pasteur, France) is human antibody RABV1 (17C7). It demonstrated neutralizing activity in 92% of cases in an experiment with an extended panel of viral isolates (25 variants) isolated in North and South America, Europe, Africa, and Asia. On this basis, it was selected from among eight antibodies with already confirmed activity against CVS-11. The determination of AS for RABV1 mAbs showed its interaction with AS III, where Asn336 played a key role in binding. At the same time, two more amino acid residues were detected (342 and 343) that also contributed to the interaction between RABV1 and the glycoprotein [44]. The 17C7 mAb from the CHO line has undergone stage III clinical trials [54].

Generalization of the information stated above clearly shows that the key characteristic of antibodies for rabies prophylaxis is their virus-neutralizing activity. The standard methods for determining this activity against RABV are the following two tests *in vitro*: fluorescent antibody virus neutralization test (FAVN) and rapid fluorescent focus inhibition test (RFFIT). The well-studied standard strains CVS-11 or ERA RABV are used as antigens. The prophylactic activities of antibodies, HRIG, and vaccines are compared during the studies *in vivo*, which are often carried out in hamsters. The ability of antibodies to block the wide range of RABV strains in studies in variants from different phylogroups, regions, and countries are determined. The most large-scale studies of neutralizing activity were carried out by the Crucell company that developed a cocktail of two human antibodies (CR57 and CR4098), by a team of researchers headed by De Benedictis [40] (human antibodies RVC-20 and RVC-58), by the World Health Organization Rabies Collaborating Centers (chimeric antibodies 62-71-3 and E559), and by the Serum Institute of India, Ltd. (human antibody RABV1). These experiments were carried out with 25-37 different strains.

Thus, the progress in molecular gene engineering and the need for antibodies in medicine have considerably influenced the vector of development of methods in antibody production. Chimeric and humanized antibodies become more and more a thing of the past and are replaced by human antibodies. Hence, the mastering and improvement of the methods of producing such antibodies becomes the most popular research trend.

Within the scope of this review, the investigation and corroboration of neutralizing activity is the most important stage in the development of preparations based on anti-RABV antibodies. It is necessary to properly select viral strains for the tests that would be most representative; it is important to determine potential mutations in ASs, due to which separate isolates escape neutralization. In addition, it is worth using HRIG as a reference preparation so that it would be possible to state with confidence the successful application of mAbs, which can effectively change the paradigm of human PEP.

Provision of effective antibody production. The important characteristics for the optimal choice of the system of production of recombinant antibodies are as follows: the ability to perform correct folding and glycosylation of proteins, productivity parameters, simplicity of purification, quality and quantity of the product, and safety of subsequent application of recombinant proteins in humans. In addition, in antibody production it is necessary to consider the time required for clinical trials and entrance to the market, as well as the product cost and legal regulation. Eukaryotic systems such as mammalian, insect, yeast, and plant cells are most promising for expression of recombinant human mAbs. Mammalian cells are used most frequently as they provide mAb folding and glycosylation maximally like human antibodies. The level of mAb production in such systems, due to optimization processes, can exceed 10-15 g/liter.

Therapeutic mAbs currently approved for clinical applications are produced in mammalian cells, generally using one of three cell lines: Chinese hamster ovary

(CHO) tumor cells, mouse nonsecretory myeloma (NS0) cells, and mouse myeloma (SP2/0) cells. The human embryonic retinoblastoma (PER.C6) line is also used for mAb production. The recombinant anti-RABV antibodies mentioned above were obtained using the CHO or PER.C6 cell lines. An exception is chimeric antibodies 62-71-3 and E559 produced in *Nicotiana benthamiana* plant cells.

The CHO cell line system has been most extensively used for the industrial production of recombinant proteins and has even surpassed some microbial systems in productivity. Nevertheless, it has certain limitations such as high sensitivity to pH changes in the medium, carbon dioxide levels, oxygen concentration, and the influence of hydrodynamic forces – all these factors considerably complicate the process of cell cultivation and scaling. The PER.C6 cell line was developed as an alternative to CHO cells for large-scale production of human recombinant mAbs. The PER.C6 line can be adapted to growth under various conditions with high level of production of recombinant proteins by the cells; however, they are also sensitive to the parameters listed above.

The cultivation of insect cells transformed by the recombinant baculovirus is a less complex process compared to the cultivation of mammalian cells. Insect cells do not need a supply of CO_2, grow at low temperature (27-28°C), and the optimal pH values of the medium for such cultures are within the range 6.1-6.5, which can be easily maintained by the growth medium buffer system. With all their "unpretentiousness", insect cells can produce no less amounts of the target product than mammalian cells and perform posttranslational modifications of the protein. However, it should be taken into consideration that the genotype of transformed insect cells is unstable if long-term cultivation is supposed. In addition to the viral genome instability, the expression system of insects is rather cost-based, and the cells are still more sensitive to the influence of hydrodynamic forces than CHO cells. Insect cell lines (HiFive, Sf-9) are more frequently used for vaccine production.

In the wide variety of plant objects that can be used in biotechnology (callus culture, suspension culture, root fibril culture, isolated embryos, organs, and protoplast culture), suspension cultures are most frequently used in antibody production due to the relative simplicity of handing and the highest yield of expressed product. BY-2 is the best-studied suspension plant cell line, which was derived in 1972. Among the drawbacks of using plant cell cultures, there is incomplete knowledge of cellular metabolism, low productivity, and heterogeneity and instability of the product. In addition, plant cultures need a special regime of long-term storage. Nevertheless, the mAbs of plant origin obtained by plant gene engineering provide a safe and economically substantiated alternative to the modern methods of antibody production in animal cells, which is favored by the cheaper plant media and bioprocess *per se*, the absence of dangerous viruses, and the presence of posttranslational modifications. The known CR57 mAb was also expressed in plant cells and then studied in comparison with the analog isolated from a human cell culture. The findings demonstrate that the CR57 mAb of plant origin has virus-neutralizing activity and affinity comparable with those of the mAb obtained in PER.C6 cells. The plant antibody is more rapidly excreted from an organism due to the presence of oligomannose N-glycans in its structure, but without any negative effect on its RABV-neutralizing properties.

The yeast and bacterial producers require the least cost and effort in the production of recombinant proteins. They are used to obtain relatively small proteins with molecular weight of no more than 100 kDa, because the level of expression of high molecular weight molecules is often rather low. An important difference between yeast and bacterial expression systems is the eukaryotic-type glycosylation, which, however, does not completely correspond to the posttranslational modifications of the above three systems (mammalian, insect, and plant cells). At present, *E. coli* cells are successfully used for obtaining antibody fragments such as Fab, scFv, and Fv; however, the production of full-size mAbs for therapeutic applications in bacterial cells seems to be impossible.

The optimal expression system for therapeutic mAbs, the majority of which are represented by full-size molecules, are mammalian or human cells providing all necessary posttranslational modifications. At the same time, when choosing an expression system to produce therapeutic preparations based on recombinant proteins, one should also take into consideration the cost of production and compare it with the possibilities of the target group of the population needing the drug. Thus, in some cases it makes sense to develop inexpensive expression systems such as yeasts and plants.

From multiyear studies of RABV structure and life cycle, considerable progress has been made in the urgent prophylaxis of this disease. The human mAb neutralizing RABV must in future substitute for the polyclonal sera ERIG and HRIG with high immunogenicity (ERIG), high cost, and limited availability (HRIG). The efficiency of application of the cocktail of neutralizing human mAbs binding to the nonoverlapping epitopes of the glycoprotein has been confirmed by experiments *in vivo* and by clinical trials.

Acknowledgments

This work was supported by the Ministry for Education and Science of the Russian Federation in the framework of the Federal Targeted Program for Research and Development in Priority Areas of Development of the Russian Scientific and Technological Complex for 2014-2020 (unique project code RFMEFI60716X0154).

REFERENCES

1. Banyard, A. C., Horton, D. L., Freuling, C., Muller, T., and Fooks, A. R. (2013) Control and prevention of canine rabies: the need for building laboratory-based surveillance capacity, *Antiviral Res.*, **98**, 357-364.
2. Baltimore, D. (1971) Expression of animal virus genomes, *Bacteriol. Rev.*, **35**, 235-241.
3. Johnson, N., Vos, A., Freuling, C., Tordo, N., Fooks, A. R., and Muller, T. (2010) Human rabies due to lyssavirus infection of bat origin, *Vet. Microbiol.*, **142**, 151-159.
4. Dietzgen, R. G., Calisher, C. H., Kurath, G., Kuzmin, I. V., Rodriguez, L. L., Stone, D. M., Tesh, R. B., Tordo, N., Walker, P. J., Wetzel, T., and Whitfield, A. E. (2011) Rhabdoviridae, in *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses* (King, A. M. Q., Adams, M. J., Carstens, E. B., and Lefkowitz, E. J., eds.) Elsevier, Oxford, pp. 686-714.
5. Lafon, M. (2005) Rabies virus receptors, *J. Neurovirol.*, **11**, 82-87.
6. Wunner, W. H., Reagan, K. J., and Koprowski, H. (1984) Characterization of saturable binding sites for rabies virus, *J. Virol.*, **50**, 691-697.
7. Cox, J. H., Dietzschold, B., and Schneider, L. G. (1977) Rabies virus glycoprotein: II. Biological and serological characterization, *Infect. Immun.*, **16**, 754-759.
8. Celis, E., Ou, D., Dietzschold, B., and Koprowski, H. (1988) Recognition of rabies and rabies-related viruses by T cells derived from human vaccine recipients, *J. Virol.*, **62**, 3128-3134.
9. Shakin-Eshleman, S. H., Remaley, A. T., Eshleman, J. R., Wunner, W. H., and Spitalnik, S. L. (1992) N-linked glycosylation of rabies virus glycoprotein. Individual sequons differ in their glycosylation efficiencies and influence on cell surface expression, *J. Biol. Chem.*, **267**, 10690-10698.
10. Gaudin, Y., Tuffereau, C., Benmansour, A., and Flamand, A. (1991) Fatty-acylation of rabies virus proteins, *Virology*, **184**, 441-444.
11. Marissen, W. E., Kramer, R. A., Rice, A., Weldon, W. C., Niezgoda, M., Faber, M., Slootstra, J. W., Meloen, R. H., Clijsters-van der Horst, M., Visser, T. J., Jongeneelen, M., Thijsse, S., Throsby, M., De Kruif, J., Rupprecht, C. E., Dietzschold, B., Goudsmit, J., and Bakker, A. B. (2005) Novel rabies virus-neutralizing epitope recognized by human monoclonal antibody: fine mapping and escape mutant analysis, *J. Virol.*, **79**, 4672-4678.
12. Prehaud, C., Coulon, P., Lafay, F., Thiers, C., and Flamand, A. (1988) Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence, *J. Virol.*, **62**, 1-7.
13. Benmansour, A., Leblois, H., Coulon, P., Tuffereau, C., Gaudin, Y., Flamand, A., and Lafay, F. (1991) Antigenicity of rabies virus glycoprotein, *J. Virol.*, **65**, 4198-4203.
14. Broughan, J. H., and Wunner, W. H. (1995) Characterization of protein involvement in rabies virus binding to BHK-21 cells, *Arch. Virol.*, **140**, 75-93.
15. Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z. (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation, *Nat. Genet.*, **38**, 228-233.
16. Ugolini, G. (2011) Rabies virus as a transneuronal tracer of neuronal connections, *Adv. Virus Res.*, **79**, 165-202.
17. Ugolini, G. (2008) Use of rabies virus as a transneuronal tracer of neuronal connections: implications for the understanding of rabies pathogenesis, *Dev. Biol. (Basel)*, **131**, 493-506.
18. Lewis, P., Fu, Y., and Lentz, T. L. (2000) Rabies virus entry at the neuromuscular junction in nerve muscle cocultures, *Muscle Nerve*, **23**, 720-730.
19. Lentz, T. L., Hawrot, E., and Wilson, P. T. (1987) Synthetic peptides corresponding to sequences of snake venom neurotoxins and rabies virus glycoprotein bind to the nicotinic acetylcholine receptor, *Proteins*, **2**, 298-307.
20. Langevin, C., Jaaro, H., Bressanelli, S., Fainzilber, M., and Tuffereau, C. (2002) Rabies virus glycoprotein (RVG) is a trimeric ligand for the N-terminal cysteine-rich domain of the mammalian p75 neurotrophin receptor, *J. Biol. Chem.*, **277**, 37655-37662.
21. Superti, F., Hauttecoeur, B., Morelec, M. J., Goldoni, P., Bizzini, B., and Tsiang, H. (1986) Involvement of gangliosides in rabies virus infection, *J. Gen. Virol.*, **67**, 47-56.
22. Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., and Hartmann, G. (2006) 5′-Triphosphate RNA is the ligand for RIG-I, *Science*, **314**, 994-997.
23. Rieder, M., and Conzelmann, K. K. (2011) Interferon in rabies virus infection, *Adv. Virus. Res.*, **79**, 91-114.
24. Lahaye, X., Vidy, A., Pomier, C., Obiang, L., Harper, F., Gaudin, Y., and Blondel, D. (2009) Functional characterization of Negri bodies (NBs) in rabies virus-infected cells: evidence that NBs are sites of viral transcription and replication, *J. Virol.*, **83**, 7948-7958.
25. Menager, P., Roux, P., Megret, F., Bourgeois, J. P., Le Sourd, A. M., Danckaert, A., Lafage, M., Prehaud, C., and Lafon, M. (2009) Toll-like receptor 3 (TLR3) plays a major role in the formation of rabies virus Negri bodies, *PLOS Pathog.*, **5**.
26. Vidy, A., El Bougrini, J., Chelbi-Alix, M. K., and Blondel, D. J. (2007) The nucleocytoplasmic rabies virus P protein counteracts interferon signaling by inhibiting both nuclear accumulation and DNA binding of STAT1, *J. Virol.*, **81**, 4255-4263.
27. Lafon, M. (2008) Immune evasion, a critical strategy for rabies virus, *Dev. Biol. Stand.*, **131**, 413-419.
28. Warrell, M. J. (2012) Current rabies vaccines and prophylaxis schedules: preventing rabies before and after exposure, *Travel Med. Infect. Dis.*, **10**, 1-15.
29. Faber, M. (2014) Recombinant rabies virus vaccines, in *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention* (Rupprecht, C. E., and Nagarajan, T., eds.) Elsevier Inc., Vol. 1, pp. 255-263.
30. Yusibov, V., Modelska, A., Steplewski, K., Agadjanyan, M., Weiner, D., Hooper, D. C., and Koprowski, H. (1997) Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1, *Proc. Natl. Acad. Sci. USA*, **94**, 5784-5788.
31. Montgomery, D. L., and Prather, K. J. (2006) Design of plasmid DNA constructs for vaccines, *Methods Mol. Med.*, **127**, 11-22.
32. Both, L., Banyard, A. C., Van Dolleweerd, C., Horton, D. L., Ma, J. K., and Fooks, A. R. (2012) Passive immunity in the prevention of rabies, *Lancet Infect. Dis.*, **12**, 397-407.
33. Fernandes, A., Kaundinya, J. O., Daftary, G., Saxena, L., Banerjee, S., and Pattnaik, P. (2008) Chromatographic

purification of equine immunoglobulin G F(ab′)$_2$ from plasma, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **876**, 109-115.
34. Meslin, F. X., Fishbein, D. B., and Matter, H. C. (1994) Rationale and prospects for rabies elimination in developing countries, *Curr. Top. Microbiol. Immunol.*, **187**, 1-26.
35. McKay, N., and Wallis, L. (2005) Rabies: a review of UK management, *J. Emerg. Med.*, **22**, 316-321.
36. World Health Organization (2013) WHO expert consultation on rabies: second report, *World Health Organ. Tech. Rep. Ser.*, **982**, 1-139.
37. Goudsmit, J., Marissen, W. E., Weldon, W. C., Niezgoda, M., Hanlon, C. A., Rice, A. B., Kruif, Jd., Dietzschold, B., Bakker, A. B., and Rupprecht, C. E. (2006) Comparison of an anti-rabies human monoclonal antibody combination with human polyclonal anti-rabies immune globulin, *J. Infect. Dis.*, **193**, 796-801.
38. Dietzschold, B., Gore, M., Casali, P., Ueki, Y., Rupprecht, C. E., Notkins, A. L., and Koprowski, H. (1990) Biological characterization of human monoclonal antibodies to rabies virus, *J. Virol.*, **64**, 3087-3090.
39. Both, L., Van Dolleweerd, C., Wright, E., Banyard, A. C., Bulmer-Thomas, B., Selden, D., Altmann, F., Fooks, A. R., and Ma, J. K. (2013) Production, characterization, and antigen specificity of recombinant 62-71-3, a candidate monoclonal antibody for rabies prophylaxis in humans, *FASEB J.*, **27**, 2055-2065.
40. De Benedictis, P., Minola, A., Rota Nodari, E., Aiello, R., Zecchin, B., Salomoni, A., Foglierini, M., Agatic, G., Vanzetta, F., Lavenir, R., Lepelletier, A., Bentley, E., Weiss, R., Cattoli, G., Capua, I., Sallusto, F., Wright, E., Lanzavecchia, A., Bourhy, H., and Corti, D. (2016) Development of broad-spectrum human monoclonal antibodies for rabies post-exposure prophylaxis, *EMBO Mol. Med.*, **8**, 407-421.
41. Tsekoa, T. L., Lotter-Stark, T., Buthelezi, S., Chakauya, E., Stoychev, S. H., Sabeta, C., Shumba, W., Phahladira, B., Hume, S., Morton, J., Rupprecht, C. E., Steinkellner, H., Pauly, M., Zeitlin, L., Whaley, K., and Chikwambaet, R. (2016) Efficient *in vitro* and *in vivo* activity of glyco-engineered plant-produced rabies monoclonal antibodies E559 and 62-71-3, *PLoS One*, **11**, 1-15.
42. Matsumoto, T., Yamada, K., Noguchi, K., Nakajima, K., Takada, K., Khawplod, P., and Nishizono, A. (2010) Isolation and characterization of novel human monoclonal antibodies possessing neutralizing ability against rabies virus, *Microbiol. Immunol.*, **54**, 673-683.
43. Kramer, R. A., Marissen, W. E., Goudsmit, J., Visser, T. J., Clijsters-Van der Horst, M., Bakker, A. Q., De Jong, M., Jongeneelen, M., Thijsse, S., Backus, H. H., Rice, A. B., Weldon, W. C., Rupprecht, C. E., Dietzschold, B., Bakker, A. B., and De Kruif, J. (2005) The human antibody repertoire specific for rabies virus glycoprotein as selected from immune libraries, *Eur. J. Immunol.*, **35**, 2131-2145.
44. Sloan, S. E., Hanlon, C., Weldon, W., Niezgoda, M., Blanton, J., Self, J., Rowley, K. J., Mandell, R. B., Babcock, G. J., Thomas, W. D., Jr., Rupprecht, C. E., and Ambrosino, D. M. (2007) Identification and characterization of a human monoclonal antibody that potently neutralizes a broad panel of rabies virus isolates, *Vaccine*, **25**, 2800-2810.
45. Zhao, X. L., Yin, J., Chen, W. Q., Jiang, M., Yang, G., and Yang, Z. H. (2008) Generation and characterization of human monoclonal antibodies to G5, a linear neutralization epitope on glycoprotein of rabies virus, by phage display technology, *Microbiol. Immunol.*, **52**, 89-93.
46. Lafon, M., Wiktor, T. J., and Macfarlan, R. I. (1983) Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies, *J. Gen. Virol.*, **64**, 843-851.
47. Muller, T., Dietzschold, B., Ertl, H., Fooks, A. R., Freuling, C., Fehlner-Gardiner, C., Kliemt, J., Meslin, F. X., Franka, R., Rupprecht, C. E., Tordo, N., Wanderler, A. I., and Kieny, M. P. (2009) Development of a mouse monoclonal antibody cocktail for post-exposure rabies prophylaxis in humans, *PLoS Negl. Trop. Dis.*, **3**, e542.
48. Kuzmina, N. A., Kuzmin, I. V., Ellison, J. A., and Rupprecht, Ch. E. (2013) Conservation of binding epitopes for monoclonal antibodies on the rabies virus glycoprotein, *J. Antivir. Antiretrovir.*, **5**, 37-43.
49. Kohler, G., and Milstein, C. (1975) Continuous cultures of fused cells secreting antibodies of predetermined specificity, *Nature*, **256**, 495-497.
50. Benevolensky, S. V., Zatsepin, S. S., Klyachko, E. V., Morozkina, E. V., Pozdnyakova, L. P., Sveshnikov, P. G., Solopova, O. N., Shemchukova, O. B., and Yagudin, O. B. (2012) The humanized antigen-binding fragments (Fab) against the rabies virus, the isolated DNA fragment encoding Fab against the rabies virus, the yeast cell transformed by the DNA fragment, and the method of obtaining Fab against the rabies virus using yeast, Patent No. RU2440412 (C2).
51. Crucell Holland BV. A Randomized Phase II Trial to Compare the Safety and Neutralizing Activity of CL184 in Combination with Rabies Vaccine vs. HRIG or Placebo in Combination with Rabies Vaccine in Healthy Adult Subjects (https://clinicaltrials.gov/show/NCT00656097), registered on September 2011.
52. Crucell Holland BV. Randomized Phase II Trial on Safety and Neutralizing Activity of CL184 and Rabies Vaccine versus Human Rabies Immune Globulin (HRIG) and Rabies Vaccine in Children and Adolescents (https://clinicaltrials.gov/show/NCT00708084), registered on March 2012.
53. Crucell Holland BV. Rabies Virus Neutralizing Activity and Safety of CL184, a Monoclonal Antibody Cocktail, in Simulated Rabies Post-exposure Prophylaxis in Healthy Adults (https://clinicaltrials.gov/ct2/show/study/NCT01228383), registered on April 2013.
54. Serum Institute of India Ltd. A Phase II/III, Randomized, Multi-Centric, Comparator-Controlled Study of the Safety and Neutralizing Activity of a Human Monoclonal Antibody to Rabies (SII RMAb) Administered in Conjunction with Rabies Vaccine for Post-exposure Prophylaxis in Patients Following Potential Rabies Exposure, CTRI/2012/05/002709 (http://www.ctri.nic.in/Clinicaltrials/pmaindet2.php?trialid=4191), registered on November 2012.