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Genetically delivered antibody protects against West Nile virus

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Received 15 July 2007; accepted 22 August 2007

Abstract

Gene-based delivery of recombinant antibody genes is a promising therapeutic strategy offering numerous advantages including sustained antibody levels, better safety profile and lower production cost. Here we describe generation of a recombinant antibody Fc-9E2 comprising a fusion protein between human Fc of IgG1 and a single-chain Fv derived from a hybridoma 9E2 secreting a mAb neutralizing West Nile virus (WNV). Fc-9E2 was shown to retain parental mAb’s specificity and WNV-neutralizing capacity. Adenovirus-mediated in vivo delivery of the antibody gene resulted in sustained Fc-9E2 serum levels leading to abrogation of lethal WNV infection in an animal model.

Keywords: WNV; Passive immunization; Recombinant antibody; Gene delivery; Adenovirus

1. Introduction

In 1999, West Nile flavivirus was introduced into the Western hemisphere and is considered an important emerging pathogen. While development of vaccines to control WNV and other flavivirus infections continues to attract considerable attention (Pugachev et al., 2003), even the most efficient flavivirus vaccines would provide a little help in treatment of ongoing infections. Transfer of specific antibodies (Ab) or immunoglobulins has been shown to abort or modify a number of Flavivirus infections (Phillpotts et al., 1987; Roehrig et al., 2001; Schlesinger et al., 1985). Passive immunization with Abs against WNV can prevent disease in animals infected with WNV (Ben-Nathan et al., 2003; Diamond et al., 2003; Engle and Diamond, 2003). While therapeutic use of human immune globulin obtained from pooled donor sera collected in regions with the high occurrence of WNV infections has been suggested (Ben-Nathan et al., 2003), such therapy is not yet widely available. Genetic engineering provides the means to generate recombinant antibodies (recAbs) in vitro by employing eukaryotic expression systems, thus expanding the therapeutic Ab repertoire. Soluble recAbs were demonstrated to bind and effectively neutralize various viral pathogens in vitro and in vivo (Prosnik et al., 2003; Wu et al., 2007). Effective protection against WNV was achieved after passive transfer of WNV specific recAbs (Gould et al., 2005; Oliphant et al., 2005; Throsby et al., 2006). However, production of clinical grade antibodies for passive immunization, either natural or engineered, is complicated. Due to high-quality standards applied to clinical grade Ab preparations, the cost of Ab production and/or purification is extremely high (Kasuya et al., 2005). An attractive alternative to passive inoculation of protective antibodies is in vivo production of therapeutic Abs by gene transfer.

Various delivery vectors, both non-viral and viral, have been developed for genetic transfer of antibody genes (Bakker et al., 2004). Although adenovirus (Ad) gene transfer vectors have been used to deliver recAbs for cancer applications (Alvarez et
al., 2000; Jiang et al., 2006; Yoshio-Hoshino et al., 2007), studies on the use of Ad vectors for Ab gene delivery are still under-represented in the treatment of infectious diseases. Kasuya et al. (2005) reported substantial survival advantage from anthrax lethal toxin achieved by an Ad vector-mediated recAb gene transfer (Kasuya et al., 2005). Recombinant Ads are attractive delivery vehicles for this application for many reasons. Ad vectors are stable; they can be easily engineered to incorporate large transgenes, their high-titer production and purification can easily be achieved using inexpensive methods. There is an extensive safety record for replication-deficient recombinant Ad in humans (NIH report, 2002). Transduction efficiency achieved using Ad vectors in vivo is unparalleled among gene delivery vectors, either non-viral or viral. The intrinsic property of replication-incompetent Ad vectors to mediate transient gene transfer looks especially attractive in the context of acute infectious disease treatment with recAbs. The vector is cleared from the organism when the disease is eliminated.

However, as an antibody molecule is a product of two gene expression, special attention should be given to design gene delivery vectors encoding recAbs. It is difficult to achieve balanced expression of two genes with a single vector. Single-chain variable fragment (scFv) is a genetically engineered, single polypeptide molecule that consists of the variable fragments of the heavy chain (VH) and the light chain (VL) of an immunoglobulin joined together by a flexible peptide linker. RecAbs can be engineered to possess new and useful properties by fusion of an scFv to other proteins, including immunoglobulin constant fragments, thereby introducing new therapeutic, physiological and/or biochemical functions. The functional activity of a successful therapeutic Ab is most likely dictated by its entire structural composition, meaning that both the antigen (Ag)-binding and the effector portions of a recombinant antibody should be preserved. Recently, Lo et al. described an approach that permits high level production of recombinant proteins in eukaryotic expression systems, whereby the protein of interest is produced as a carboxy-terminal fusion protein with an immunoglobulin constant fragment (fragment crystallizable, Fc) (Lo et al., 1998). By swapping the Fc portions of recAbs it is possible to tailor their effector functions. We further developed this approach by designing and producing a recombinant antibody molecule, that includes an scFv as the C-terminal fusion with the IgG Fc fragment. It has been demonstrated that the recombinant antibody in this configuration retains its binding properties (Korokhov et al., 2003).

In the current study we describe Ad-mediated gene transfer of a WNV-neutralizing recAb gene resulting in high and sustained levels of protective recAb in vivo. We demonstrate that a single injection of such engineered Ad prevents the development of lethal WNV infection in mice.

2. Materials and methods

2.1. Cells and viruses

WNV strains were grown on Vero cells (ATCC CRL-1586) at 37°C in a humidified atmosphere containing 5% CO2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% FetalClone III (FClIII; Hyclone, Logan, UT) and 1× antibiotic–antimycotic mixture (Invitrogen, Carlsbad, CA). Strain Vlg99-27889 (Lvov et al., 2000) was obtained from the State Collection of Virus Strains (Ivanovskii Institute of Virology, Russian Academy of Medical Sciences). Strain Eg101 (Melmick et al., 1951) was obtained from Dr. L. Zeng (CBER, Bethesda, MD). A 385–99 isolate of the NY99 strain (Tesh et al., 2002) was provided by Dr. R. Tesh (Galveston, TX). Recombinant Ad vectors were rescued and propagated on HEK-293 cells (ATCC CRL-1573). Human lung adenocarcinoma A549 cells (ATCC CCL-185) were used for in vitro production of the Fc-9E2 antibody. These cells were grown in DMEM/F12 medium supplemented with 10% FCS and 1× antibiotic–antimycotic mixture.

2.2. Gene engineering and Fc-9E2 production/purification

A fragment encoding the C-terminal portion of the envelope (E) protein of Vlg99 WNV (WNVEC; aa 551-743, GenBank #AAP22089) was PCR-amplified from a construct described in (Razumov et al., 2005) and cloned in the pET21a expression vector (Novagen, Madison, WI). The protein was produced in BL21(DE)pLySs bacteria and purified by immobilized metal affinity chromatography (IMAC) using TALON resin (Clontech, Palo Alto, CA) followed by dialysis against PBS.

To generate scFv 9E2, total RNA was isolated from a hybridoma cell line 9E2 secreting neutralizing monoclonal antibody (mAb) to WNV envelope protein using RNeasy kit (Qiagen, Valencia, CA). Monoclonal antibody produced by this hybridoma demonstrated a high neutralization activity against several WNV isolates. MAb 9E2 recognizes the C-terminal portion of WNV E protein (Domain III) (Razumov et al., 2005). Coding DNA was synthesized using Omniscript RT kit (Qiagen, Valencia, CA) and was used for PCR amplification of VH and VL gene fragments with a set of published primers (Breitling et al., 2001). The PCR products were ligated into a phagemid vector pSEX81 (Breitling et al., 1991). Escherichia coli cells bearing pSEX/9E2 were infected with M13KO7 helper phage resulting in bacteriophages displaying scFv 9E2 variants. This phage-scFv mini-library was screened once using a purified WNVEC, as described (Kontermann, 2001). A positive phage clone was identified by ELISA using purified WNVEC. Nucleotide sequence of scFv 9E2 was determined by DNA sequencing of phage replicative form DNA. The scFv gene was re-cloned into a bacterial expression vector pOPE101 (Dubel et al., 1993) featuring a c-myc detection tag and the His6 purification tag.

Assembly ofFc-9E2-coding DNA was performed as follows. The hinge, CH2 and CH3 domains (collectively called the Fc, or fragment crystallizable) of the human immunoglobulin gamma-1 gene was PCR-amplified from a cDNA clone (Open Biosystems, Huntsville, AL; GenBank #BC073782) introducing a single Cys(248)Ser (according to AAH73782) amino acid substitution in the hinge region. That cysteine residue normally forms a disulfide bond with the light chain. The Fc gene fragment was cloned into pSecTag2a vector (Invitrogen) in frame.
with the murine Ig kappa-chain V-J2-C signal peptide sequence. Gene of scFv 9E2 was PCR amplified and cloned in frame with the leader/Fc, generating pSecTag/Fc-9E2.

The entire expression cassette including the CMV promoter, Fc-9E2 and bovine growth hormone (BGH) polyadenylation signal was re-cloned into the pShuttle vector (Stratagene, La Jolla, CA) generating pShuttle/CMV/Fc-9E2. To generate a recombinant Ad genome, the AdEasy Adenoviral Vector System (Stratagene) was used in accordance with the instruction manual. The recombinant Ad virus genome was transfected to HEK-293 cells, and recombinant Ad/Fc-9E2 virus was rescued and mass-produced followed by CsCl gradient purification. The biological virus titers were determined per AdEasy manual as plaque-forming units (pfu) on 293 cells. Virus particle (v.p.) titer was determined spectrophotometrically (Maizel et al., 1968), using a conversion factor of $1.1 \times 10^{12}$ v.p. per one absorbance unit at 260 nm. Ad/Fc-G19—an Ad vector encoding an irrelevant recAb, recognizing the spike protein of SARS coronavirus, has been generated similarly. The v.p. to pfu ratio in the Ad preparations used in this study was ~10:1.

For Fc-9E2 production, recombinant Ad/Fc-9E2 was used as an expression system. A549 cells were transduced with the Ad/Fc-9E2 at the multiplicity of infection (MOI) of 100 v.p./cell. Infection was carried out for 1 h at 37°C followed by medium replacement. Medium was replaced every 3 days, up to day 12 post-infection, while collecting the supernatant. Recombinant Fc-9E2 protein was purified using Protein A Antibody Purification Kit (Sigma, St. Louis, MO). Protein concentration was determined by the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine gamma globulin as a standard.

2.3. RecAb characterization

To test Fc-9E2 production in Ad5/Fc-9E2-transduced A549 cells, aliquots of the purified recAb were separated on 5–15% gradient SDS PAAG. The samples were run in either boiled or unboiled form (to assay for the dimer formation; 2-mercaptoethanol (ME) was also omitted in the unboiled sample), followed by Western blot with horseradish peroxidase (HRP)-labeled goat anti-human IgG Abs (DAKO, Carpinteria, CA) and diaminobenzidine (DAB, Sigma) staining.

Binding of Fc-9E2 to WNV virions was examined by indirect ELISA. As capturing Abs, anti WNV mAb 5H6 (Razumov et al., 2005), or an irrelevant mAb as negative control, were used at 1.2 μg/well. The wells were then incubated with WNV-infected (Eg-101) Vero cell lysate (triple freezing-thawing followed by sonication) followed by Fc-9E2, in dilutions. Binding of Fc-9E2 to WNVEC was tested in ELISA: WNVEC was absorbed on plastic of a 96-well microplate at 400 ng/well. Aliquots of Fc-9E2 dilutions were added to the wells followed by HRP-labeled goat anti-human IgG Abs and orthophenylene diamine (OPD, Sigma) staining. Fc-9E2 interaction with WNV E protein was also examined in radioimmunoprecipitation (RIP) as described previously (Yamshchikov et al., 1997). Briefly, BHK cells transfected with the W956 infectious clone (Yamshchikov et al., 2004) were labeled at 15 h post-infection with 100 μCi of $^{35}$S Met (EasyTag, Perkin-Elmer, Waltham, MA) and chased for 5 h in the complete growth medium. Virus-specific proteins were immunoprecipitated from the culture medium and cell lysate using either a WNV E protein-specific mAb E31 (Oliphant et al., 2006), or purified Fc-9E2, and protein-A agarose. Proteins were separated using 12% SDS-PAGE and visualized using an X-ray film (Kodak BioMax, Fisher, Pittsburgh, PA).

The WNV neutralization assay was performed on Vero cells using the 96-well micro-plaque reduction neutralization test (PRNT) as reported earlier (Yamshchikov et al., 2004, 2005).

2.4. Animal studies

Mice were maintained according to the American Association for Accreditation of Laboratory Animal Care guidelines. Animal protocols were reviewed and approved by the Kansas University’s Institutional Animal Care and Use Committee.

To determine Fc-9E2 circulation levels resulted from Ad injection, female BALB/c mice (Charles River Laboratories; Wilmington, MA) at 6 weeks of age were inoculated intraperitoneally (i.p.) with either $10^{6}$ or $10^{5}$ pfu of Ad/Fc-9E2. Sera samples were collected at days 1, 2, 3, 4, 5, 7, 14 and 21 post-inoculation and kept at −20°C until analysis. Three mice per time point were sacrificed per each viral dose. Fc-9E2 concentrations in individual sera were determined using Human IgG ELISA Quantitation Kit (Bethyl Labs; Montgomery, TX).

For animal protection studies, 4-week-old female Swiss Webster outbred mice (Harlan, Indianapolis, IN) were inoculated i.p. with either Ad/Fc-9E2 or Ad/Fc-G19 encoding an irrelevant recAb. Groups of six mice were inoculated with corresponding recAd at $10^{6}$ pfu/mouse: (i) 24 h before; (ii) simultaneously with; and (iii) 24 h after i.p. challenge with WNV NY99 (200 pfu/mouse). A control group of six mice was infected with 200 pfu of NY99. The mice were monitored for survival for 14 days. Blood samples were collected at indicated time points and tested for Fc-9E2 circulation and anti-WNV mouse IgM. Fc-9E2 and mouse anti-WNV IgM levels in mouse sera from protection studies were determined in ELISA using semi-purified viral NY99 antigen (NY99 WNV pelleted through 20% sucrose cushion; solubilized with 0.5% Triton-X100 in PBS) as described earlier (Yamshchikov et al., 2004, 2005) using either goat anti-human IgG or goat anti-mouse IgM HRP-labeled secondary antibodies. The color reaction was developed with tetrathymethyl-benzidine (TMB, Sigma).

2.5. Statistics

Differences in the survival of mice were analyzed using the Kaplan–Meier method, and groups were compared using the log-rank test using the GraphPad Prism software. Antibody titers measured in challenge experiments were compared by Repeated Measures ANOVA analysis after natural-logarithmic (ln) transformation of each value, where $P < 0.05$ was considered significant.
3. Results

3.1. Characterization of anti-WNV recAbs

The aim of this study was to test whether gene-based in vivo production of pathogen-specific neutralizing recAbs can be useful in the treatment of infectious diseases. To achieve that we have generated a recombinant Ad vector encoding secreted recAb Fc-9E2 using the genetic material from a hybridoma cell line secreting monoclonal antibody 9E2 (Fig. 1a) This mAb displayed a strong neutralizing activity against different strains of WNV (Razumov et al., 2005). Coding DNA of the VH and VL portions of mAb 9E2 were PCR-amplified and cloned to encode scFv 9E2 (Fig. 1b). The Fc-9E2 (Fig. 1c) expression cassette was then generated that included a gene of secreted Fc-9E2 (the mouse Ig kappa leader peptide followed by human IgG1 Fc and scFv 9E2 ORF) upstream of the SV40 polyadenylation signal. The entire cassette controlled by the CMV promoter was incorporated into an Ad vector. The resulting recombinant virus Ad/Fc-9E2 (Fig. 1d) was used in this study for both in vitro protein production and for in vivo recAb gene delivery (Fig. 1e). We anticipated that after inoculation of animals with Ad/Fc-9E2 sustained levels of WNV-neutralizing recAbs in blood will confer protection against otherwise lethal WNV infection. Sequences of scFv 9E2 VH and VL were deposited in GenBank under accession numbers EF571851 and EF571852, respectively.

ScFv 9E2 produced in bacteria and purified from periplasmic extract was shown to recognize purified C-terminal fragment of WNV E protein–WNVEC in ELISA (data not shown). Fc-9E2 produced in vitro was purified from the culture supernatant of Ad/Fc-9E2-transduced A549 cells using protein A chromatography. The yield of Ad-mediated recAb production was ~5 mg/L. Western blot of purified Fc-9E2 stained with anti-human IgG Abs (Fig. 2a) revealed a specific band of ~55 kDa (left lane) corresponding to the recAb’s monomeric form under reducing conditions (ME, 100 °C). Unreduced Fc-9E2 (no ME, no boiling) migrated as a ~110 kDa band (right lane), which corresponds to the dimeric form of the recAb. Purified

Fig. 1. Schematic outline of the study. Genetic material form a hybridoma secreting the WNV-neutralizing mAb 9E2 (a) was used to generate scFv 9E2 (b) and then Fc-9E2 (c) coding sequences. Expression cassette comprising Fc-9E2 ORF, preceded by mouse kappa chain leader peptide sequence and followed by a polyadenylation signal, placed under the control of the CMV promoter was introduced into an Ad genome, which was further used to generate recombinant Ad/Fc-9E2 (d). The vector was used to inoculate mice (e) achieving Fc-9E2 in vivo production resulting in WNV neutralization and animal protection.

Fig. 2. Characterization of Fc-9E2. (a) Western blot of purified Fc-9E2. A549 cells were transduced with the Ad/Fc-9E2. The recAb was protein A-purified from culture supernatant. Aliquots of the purified recAb were separated by SDS-PAGE followed by Western blot with HRP-labeled goat anti-human IgG Abs and DAB staining. (R) Sample in reducing (2-ME, boiling) conditions; (UR) sample in unreducing (no 2-ME, no boiling) conditions. (b) Indirect ELISA with WNV-infected cell lysate. Anti WNV mAb 5H6 (squares) or an irrelevant mAb (diamonds) were used as capturing Abs. The wells were incubated with WNV-infected (Eg-101) Vero cell lysate followed by Fc-9E2, anti-human IgG/HRP and DAB staining. (c) Radioimmunoprecipitation with the lysate of BHK cells transfected with WN956 infectious clone. Cell lysate was incubated with Fc-9E2 (lane 1) and WNV E protein-specific mAb E31 (lane 2) followed by precipitation with protein A agarose. Proteins were separated by 12% SDS-PAGE and visualized on X-ray film. (d) Fc-9E2 ELISA with WNVEC. Fc-9E2 was incubated with either WNVEC (squares) or casein (diamonds) followed by HRP-labeled goat anti-human IgG Abs and DAB staining.
Fc-9E2 was demonstrated to specifically bind WNV virions (strain Eg101) in indirect ELISA (Fig. 2b). Purified Fc-9E2 specifically immunoprecipitated WNV E protein from lysates of WNV infected radiolabeled BHK cells (Fig. 2c). It was shown previously (Razumov et al., 2005) that mAb 9E2 interacted with the C-terminal portion of WNV E protein. ELISA with the corresponding antigen WNVEC demonstrated that Fc-9E2 retained specific binding to this antigen (Fig. 2d). This was further confirmed by Western blot with purified WNVEC (data not shown).

Next we examined the ability of purified Fc-9E2 to neutralize WNV in vitro using plaque reduction-neutralization test. As shown in Table 1, Fc-9E2 efficiently neutralized infectivity of WNV strains NY99 (isolate 385-99) and Eg101. As expected, Fc-9E2 also neutralized WNV strain Vlg99-27889 (data not shown).

Thus, an adenovirus encoding recombinant antibody Fc-9E2 achieved a high level of recAb production in cell culture. Fc-9E2 was demonstrated to form dimers, retained the parental mAb’s specificity to the C-terminal portion of the WNV E protein and efficiently neutralized different isolates of WNV.

### 3.2. RecAb gene transfer in vivo

We anticipated that inoculation of animals with Ad/Fc-9E2 will result in sustained levels of recAbs in the circulation, which would last for a time sufficient to completely abrogate WNV infection. In order to evaluate these parameters we injected Ad/Fc-9E2 into mice and monitored human IgG Fc in the blood during 3 weeks after inoculation. Two groups of mice were injected i.p. with Ad/Fc-9E2 at the doses of $10^8$ and $10^9$ pfu per mouse. The results shown in Fig. 3 demonstrate that at the dose of $10^8$ pfu/mouse, recAb was already detectable in the blood at 24 h post-inoculation and reached maximum levels at days 3–5 post-inoculation. Detectable recAb levels were still observed at 14 and even 21 days post-inoculation. The dose $10^8$ pfu/mouse resulted in lower overall recAb levels and an earlier decrease beyond the detection limit.

### 3.3. Animal protection

The ultimate goal of our study was to demonstrate that Ad-mediated recAb gene delivery resulting in sustained circulation level of Fc-9E2 will abrogate lethal WNV infection in animal model. To test that, mice were infected with 200 pfu of WNV NY99 (10 LD$_{50}$ (Yamshchikov et al., 2004)) and treated with Ad/Fc-9E2, or Ad/Fc-G19 encoding an irrelevant recAb, at days $-1, 0$ (preventive studies), and $+1$ (therapeutic study), in regard to the NY99 challenge. As shown in Fig. 4a, Ad-mediated in vivo production of anti WNV Fc-9E2 resulted in a remarkable increase in survival of animals compared to those which received the Ad encoding an irrelevant recAb (Fig. 4b). Survival of 83.3%
(5/6) animals was achieved when Ad/Fc-9E2 was administered 24 h before WNV challenge. The survival advantage in this group over the group not receiving Ad vectors was statistically significant \((P = 0.0289; \text{log-rank test})\). All mice survived when Ad vector was given either simultaneously with, or 24 h after the challenge. Survival in these groups was significantly higher than in control group \((P = 0.0042; \text{log-rank test})\). Serum levels of Fc-9E2 after WNV challenge were determined by ELISA using the NY99 coating antigen. As expected, Fc-9E2 was clearly detectable during the monitored interval (Fig. 5a–c). An indication of establishment and progression of active WNV infection is the production of host IgM at early stages of the process (Tardei et al., 2000). We measured mouse anti-WNV IgM in the blood during the course of treatment. As shown in Fig. 5d–f, levels of WNV-specific IgM did not rise in the animals receiving Ad/Fc-9E2, thus indicating an efficient suppression of infection. In contrast, mice receiving control Ad showed significantly higher host IgM levels, both for animals receiving Ads 24 h before \((P = 0.0008)\), and at the time of the challenge \((P = 0.00006)\). No statistically significant difference was observed between the mice groups that received Ads 24 h after the challenge, likely indicating the establishment of initial WNV infection that later was abrogated by recAb gene transfer resulting in increased survival. All the comparisons were made at day 7 post-WNV challenge.

4. Discussion

The use of antibody-based therapies against infectious agents in routine clinical practice, although very efficient in many applications, is limited by several factors including safety issues and cost. Therapeutic antibodies are among the most expensive drugs used in clinical practice (Kasuya et al., 2005). The high cost of Ig preparations is related to the fact that these reagents require special handling and are costly to obtain, produce, and maintain. Immunoglobulins derived from immune donors may contain adventitious agents (including yet unknown ones) or may be potentially bio-hazardous otherwise, since the 100% purity cannot be achieved. In addition, in vitro production of Abs, especially the full size, two-chain Abs, requires tedious optimization and the yields are often low. We show in this study that there is an alternative to purified Ab preparations for passive immunization. Gene-based delivery of protective recAbs offers a number of advantages. First, the recAbs are produced in vivo and therefore are properly processed by the host mechanisms. Second, there is no need for recAb purification, although clinical vector preparations have to be produced according to the accepted criteria. Third, the recAb gene expression is maintained over time, thus eliminating the need for repeated injections of Abs to maintain therapeutic concentrations. An important safety feature of the proposed treatment is that we used a replication incompetent Ad vector for recAb gene delivery. The ability of replication-deficient Ad vectors to mediate transient gene transfer looks especially attractive in the context of acute infectious disease treatment with recAbs.

The strategy described here is highly generalizable by swapping the pathogen binding/neutralizing portion of the recAb it is possible to generate therapeutics against infectious agents for which passive immunization was proven to be effective. This approach can be used for at-risk individuals such as laboratory personnel, or during a possible bio-terrorism attack. Pre-existing anti Ad immunity resulted from previous exposure to Ad either through natural infection or administration of a vector may affect applications in which long-term expression is necessary and which will require re-administration of vector following the eventual loss of therapeutic transgene expression. We show here that a single dose Ad-recAb treatment abrogated WNV infection in laboratory animals. Nevertheless, should a need for repeated Ad administrations arise, several studies have documented effective re-administration in certain applications. For example, in a phase I/II trial for recurrent ovarian cancer where intraperitoneal re-administration was used, transgene expression was measurable in 17 of 20 samples obtained after two or three cycles (Buller et al., 2002). In another phase I clinical, 5 out of 6 tested patients’ sera were positive for anti Ad antibodies but that did not result in reduction of the transgene expression after i.p. administration of Ad21 encoding for an intrabody (Alvarez et al., 2000). Therefore, it should be recognized that the ability to effectively repeat administer Ad vectors is dose-dependent and site-specific.

In the present study we describe generation of a recAb Fc-9E2 comprising a fusion protein between the Fc portion of human IgG1 linked to an scFv derived from a hybridoma 9E2 that secretes a mAb capable of neutralizing different isolates.
of WNV. MAb 9E2 was shown previously to recognize the C-terminal portion (domain III) of WNV E protein (Razumov et al., 2005). Domain III is the putative receptor binding domain of the virus (Crill and Roehrig, 2001) and an important target for neutralizing antibodies (Beasley and Barrett, 2002; Oliphant et al., 2005; Razumov et al., 2005; Sanchez et al., 2005). Neutralizing Abs that bind to the E protein prevent infection of cells by blocking attachment of virus to cellular receptors and attachment factors (Crill and Roehrig, 2001). Recent data suggests that DIII-specific neutralizing antibodies may also be inhibiting at a post-attachment step (Nybakken et al., 2006). A replication-deficient recombinant Ad vector has been generated to encode Fc-9E2 under the control of powerful CMV promoter. The recombinant Ad mediated sustained levels of the recAb production after systemic administration into mice, which lasted for at least 3 weeks post-inoculation. Our expectation that gene-based in vivo expression of recAbs may serve as an efficient treatment of an acute infectious process was confirmed in WNV challenge studies in mice treated with Ad/Fc-9E2. The experiments were performed using three regimens: (i) with the Ad vector given 1 day before the lethal WNV challenge; (ii) with Ad vector given simultaneous with the lethal WNV challenge; and (iii) with Ad vector given 1 day after the lethal WNV challenge. An outstanding animal protection was observed after a single injection of Ad/Fc-9E2 in all three treatments groups, which was contrasted by the high mortality in control groups that received Ad/Fc-G19 encoding an irrelevant recAb. All mice have survived in the groups receiving the Ad encoding the WNV-specific recAb simultaneously with and 24h after the lethal WNV challenge. Treatments with Ad/Fc-9E2 in the WNV challenged animals resulted in significant and sustained specific recAb levels in sera of inoculated mice as determined by WNV-specific ELISA. Since WNV-specific IgM responses were not detected in these animals, we concluded that such treatment effectively suppressed the development of active WNV infection. Thus, a single dose of recombinant Ad vector that mediated WNV-specific recAb gene delivery resulted in abrogation of acute WNV infection, both in prophylactic and in therapeutic treatment regiments. To our knowledge, this is the first demonstration of Ad mediated recAb gene transfer that resulted in abrogation of an acute infectious process. Ablation of infection is supported by the observed suppression of early IgM response in animals that survived the lethal challenge. Earlier, Kasuya et al. (2005) had demonstrated an Ad mediated in vivo neutralization of anthrax toxin (Kasuya et al., 2005) but not infection caused by an active pathogen.

The timing of passive Ab administration relative to the development of clinical symptoms is the determining factor for the treatment’s efficacy. Elimination of WNV disease was shown to be successful when pre-existing Abs were administered even at day 5 after virus challenge (Oliphant et al., 2005). We demonstrate here that recAb delivered 1 day after the challenge abrogated WNV infection. Determination of Ad-mediated Fc-9E2 efficacy at later WNV infection stages is the subject of our ongoing studies.

Beside direct pathogen neutralization in vivo, protective antibodies (even non-neutralizing in vitro) can utilize other mechanisms to eliminate pathogens and abrogate disease, e.g. antibody-dependent and complement-mediated cellular cytotoxicity-functions attributed to the Fc. The key event in the former mechanism is interaction of IgG attached to infected cells to Fcγ receptor III. Residues Leu-Leu-Gly-Gly-Pro of the lower hinge of IgG1 have been implicated in the receptor binding (Lund et al., 1991), and since they are preserved in Fc-9E2, this function should be present in the described construct. Residues D270, K322, P329, and P331 which are responsible for C1q complement sub-component binding to IgG (Idusogie et al., 2000) are also preserved in our construct and therefore, Fc-9E2 should have the latter function, too. Neonatal Fc receptor (FcRn) plays important role in regulation of IgG circulation half-life favoring recycling and/or transcytosis rather than catabolism (Ghetie and Ward, 1997). The residues responsible for Fc interaction to FcRn have been identified in CH2 and CH3 domains (Kim et al., 1999) and are as well preserved in our construct. To experimentally confirm that these important effector functions are indeed retained in the construct, we are planning detailed studies of mechanisms of Fc-9E2-mediated anti-WNV protection.

Acknowledgements

Authors thank Dr. Alla Kachko for technical assistance. This work was supported by NIH grants 5R21 AI054538, 5U54 AI057157, 1R03 AI53084 and 1R01 AI049258. The authors declare no competing interests.

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