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Chimeric vaccine composed of viral peptide and mammalian heat-shock protein 60 peptide protects against West Nile virus challenge

Orly Gershoni-Yahalom,[1] Shimon Landes,[1] Smadar Kleiman-Shoval,[1] David Ben-Nathan,[2] Michal Kam,[3] Bat-El Lachmi,[2] Yevgeny Khinich,[4] Michael Simanov,[4] Itzhak Samina,[4] Anat Eitan,[3] Irun R. Cohen,[5] Bracha Rager-Zisman[1] and Angel Porgador[1]

[1]*The Shraga Segal Department of Microbiology and Immunology, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva,* [2]*Department of Infectious Diseases, Israel Institute for Biological Research, Ness-Ziona,* [3]*VacciGuard Ltd., Misgav Business Park, M.P. Misgav,* [4] *Kimron Veterinary Institute, Virology, Beit Dagan, and* [5]*Department of Immunology, Weizmann Institute of Science, Rehovot, Israel*

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Correspondence: A. Porgador and B. Rager-Zisman, Department of Microbiology and Immunology, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva 84105, Israel.
Email: angel@bgu.ac.il; rager@netvision.net.il
Senior author: Angel Porgador,
email: angel@bgu.ac.il

Summary

The protective efficacy and immunogenicity of a chimeric peptide against West Nile virus (WNV) was evaluated. This virus is the aetiological agent of West Nile fever, which has recently emerged in the western hemisphere. The rapid spread of WNV throughout North America, as well as the constantly changing epidemiology and transmission of the virus by blood transfusion and transplantation, have raised major public-health concerns. Currently, there are no effective treatments for WNV or vaccine for human use. We previously identified a novel, continuous B-cell epitope from domain III of the WNV envelope protein, termed Ep15. To test whether this epitope can protect against WNV infection, we synthesized a linear chimeric peptide composed of Ep15 and the heat-shock protein 60 peptide, p458. The p458 peptide is an effective carrier peptide for subunit vaccines against other infectious agents. We now report that mice immunized with the chimeric peptide, p458-Ep15, were resistant to lethal challenges with three different WNV strains. Moreover, their brains were free of viral genome and infectious virus. Mice immunized with Ep15 alone or with p431-Ep15, a control conjugate, were not protected. The chimeric p458-Ep15 peptide induced WNV-specific immunoglobulin G antibodies that neutralized the virus and induced the secretion of interferon-γ *in vitro*. Challenge of chimeric peptide-immunized mice considerably enhanced WNV-specific neutralizing antibodies. We conclude that this chimeric peptide can be used for formulation of a human vaccine against WNV.

Keywords: chimeric peptide; E protein; heat-shock protein 60; vaccine; West Nile virus

Introduction

West Nile Virus (WNV), the aetiological agent of West Nile fever, is a single positive-stranded RNA virus that is taxonomically classified within the *Flaviviridae* family.[1] The virus is maintained in nature in a mosquito-borne cycle, with humans, horses and other mammals serving as incidental hosts.[2]

First isolated in Uganda in 1937,[3] WNV was historically known to be endemic in Africa, West Asia and the Middle East.[4,5] Since 1999 the epidemiology has changed and the virus has emerged in the western hemisphere, including the USA.[6–9] Following its introduction into the USA in 1999, WNV has spread rapidly across the North American continent, infecting a wide range of avian and mammalian species, including humans.[10–12] It has also been shown that WNV can be transmitted between humans through blood transfusions, organ transplantation, intrauterine transmission, laboratory-acquired infection and breastfeeding.[13–16]

Clearly, there is an urgent need for an effective WNV vaccine to protect populations at risk. Although a number of different WNV vaccines are now at various stages of

development and testing,[17–23] no licensed human vaccine for the prevention of WNV infection is yet available. Antibodies play a major role in the protection and recovery from WNV and indeed, currently, the only effective manner to provide immediate resistance to WNV is by the passive administration of WNV-specific antibodies.[24–27]

Domain III of the WNV envelope protein (E-DIII) is responsible for the recognition and attachment of the virus to its cellular receptor,[28] and therefore it is an ideal vaccine target. It has been shown that neutralizing antibodies react with epitopes that are localized in the E-DIII region.[29–31]

Ep15 is a continuous B-cell epitope derived from WNV E-DIII (amino acids 355–369).[32] The sequence of Ep15 in different WNV strains is nearly identical,[7,30] and evidence for the recognition of the Ep15 region by neutralizing anti-WNV antibodies has been reported.[33–35]

We have previously investigated heat-shock protein 60 (hsp 60) -p458, a peptide derived from the hsp 60 molecule, as a carrier T-cell epitope for foreign antigens in conjugate vaccines.[36–40] The hsp 60 belongs to a family of chaperone molecules highly conserved throughout evolution. Apparently, no cell can exist without the ability to express hsp 60 upon encountering stress.[41] A similar hsp 60 molecule is present in all cells, both prokaryotic and eukaryotic. As a consequence, hsp 60 is shared by a host and its parasites, and universally expressed in inflammation. Heat-shock protein 60 is well recognized by the immune system; it binds to toll-like receptor 4 (TLR4),[42,43] serves as a link between innate and adaptive immunity[44,45] and is part of the set of self-molecules for which autoimmunity naturally exists. Heat-shock, interferon-γ (IFN-γ), bacterial or viral infection, and inflammation all result in the presentation of endogenous –hsp 60 epitopes on major histocompatibility complex class II molecules, leading to the activation of hsp 60-specific T cells, even in healthy individuals.[46–49] Natural T-cell reactivity to the hsp 60 self-epitope, p458, in conjugated vaccines can be mobilized to induce resistance to a lethal infection. Indeed, we have shown the efficacy of this immunization approach against different bacterial and viral models.[36–40] Finally, hsp 60-p458 was also effective as a carrier peptide for bacterial antigens in elderly mice and was able to induce long-lasting anti-pathogenic memory.[38]

Here, we report a construction of a chimeric subunit peptide capable of inducing WNV-specific antibodies and IFN-γ responses while conferring protective efficacy against a lethal challenge of WNV.

Materials and methods

Mice

Fourteen-day-old female BALB/c mice (10–12 g body weight) were purchased from Harlan Olac (Jerusalem, IL). The mice were maintained under specific pathogen-free conditions and allowed to adjust to the facility for 7 days before experiments were performed. The animals were used at the age of 3 weeks, unless otherwise stated. Age- and sex-matched animals served as controls. Mice were maintained in isolation cages and were fed and watered *ad libitum*. Experiments with the mice were approved and performed according to the guidelines of the Faculty of Health Sciences, Animal Safety Committee, Ben Gurion University.

Cells

The Vero cell line, derived from African Green Monkey (ATCC® number: CCL-81), was used. The cells were grown in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (1% Pen-Strep), 2 mM L-glutamine and 10% fetal bovine serum (FBS). The cells were maintained in a humidified atmosphere at 37° in 5% CO_2 and were used for growing viral stocks, virus titrations and antibody neutralization assays.

Peptides

Peptides were synthesized by Sigma-Aldrich (Rehovot, IL) and American-Peptide (Sunnyvale, CA). Peptide purity was ascertained by analytical reverse-phase high-performance liquid chromatography and amino acid analysis and assessed as > 95%. The following four peptides were synthesized and studied: (i) Ep15 (derived from the EIII domain of WNV) – *LVTVNPFVSVATANS*; (ii) p458 (derived from murine hsp 60), previously reported to serve as a carrier peptide for subunit vaccines[36–40] – NEDQKIGIEIIKRALKI; (iii) the chimeric peptide p458-Ep15 (linearly synthesized) – NEDQKIGIEIIKRALKI*LVTVNPFVSVATANS*; and (iv) p431-Ep15, a negative control peptide for the effect of p458. The p431 portion corresponds to a mycobacterial hsp 60-derived peptide, lacking val 442 (this mycobacterial peptide is homologous in sequence to murine p458 but does not elicit a $CD4^+$-dependent immune response or induces an antibody response against itself or p458) – EGDEATGANIKVALEA*LVTVNPFVSVATANS*.

WNV strains and WNV antigen

West Nile virus strain ISR98 was isolated in 1998 from the brain of a dead goose in Israel in 1998 and has been previously described.[50] The NY99 strain of WNV was isolated from a human case of WNV infection and is closely related (high DNA homology) to the ISR98 strain.[6] The Goldblum strain of WNV was isolated in Israel from a human case of WNV infection.[51] The presence of signature amino acid motifs indicated that all of the above strains belonged to viral lineage I.[3,17,52] The WNV antigen was prepared as previously described.[24]

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Virus titrations

Virus plaque assays or 50% tissue culture infective dose ($TCID_{50}$) titration (for ISR98) were performed on Vero cell monolayers, as previously described.[53] Titres of viral stocks were expressed as plaque-forming units (PFU) per ml. The titres of the viral stocks were: ISR98 2×10^7 PFU/ml, NY99 5×10^8 PFU/ml and Goldblum 10^8 PFU/ml. A single stock of each virus strain was prepared in Vero cells, divided into aliquots, stored at −70° and was used throughout this study.

Immunization of mice and WNV challenge

We first performed dose escalation of the hsp 60-p458 peptide and found that 15 μg/dose is the optimal dosage. Therefore, the initial dose of the p458-Ep15 was calculated as 25 μg, which is equimolar to 15 μg p458. To study the protective efficacy of the chimeric peptide, 3-week-old mice were immunized intrafootpad three times with a 7-day interval between each injection. The immunizing dose of each peptide was equimolar to 25 μg of p458-Ep15. All peptides were emulsified in incomplete Freund's adjuvant (IFA) and injected at 50 μl/mouse. Seven days after the last immunization, the mice were inoculated intraperitoneally with a lethal dose of three different WNV strains and mortality was monitored for 21 days later.

To study the immunogenicity of the chimeric peptide, 3-week-old mice were immunized as described above. Seven or 10 days after the last immunization, the mice were bled and killed. Spleens were harvested and splenocyte cultures were prepared and tested for IFN-γ secretion as previously described.[39] Blood samples were centrifuged and sera were collected, pooled and tested for the presence of WNV-specific antibodies by enzyme-linked immunosorbent assay (ELISA) and neutralization assays.

Determination of viral load in brain tissue of infected mice

Brains were removed from infected or immunized and challenged mice, and 10% (weight/volume) homogenates were prepared in DMEM–10% dimethylsulphoxide. The homogenates were then aliquoted and stored at −70° until further analysis. Viral levels were determined by plaque titration on Vero cell monolayers as previously described[53] and expressed as number of pfu/0·1 g brain tissue.

RNA extraction and reverse transcription–polymerase chain reaction

RNA was extracted from mouse brain tissues using an RNEasy midi kit (Qiagen, Hilden, Germany), and reverse transcription–polymerase chain reaction (RT-PCR) was performed on the RNA extracts using Endo-Free reverse transcriptase (Ambion, Huntingdon, UK) and Biomix-Red (Bioline, London, UK). Primers for WNV E protein (5′-ACGAAGTGGCCATTTTTGTC-3′/5′TTGATGCAGAG CTCCCTCTT-3′) were chosen using the PRIMER3 program (Whitehead Institute for Biomedical Research, Cambridge MA). The PCR products were electrophoretically separated on 1·5% agarose gels, stained with ethidium bromide and imaged using an Imagechem 5500 CCD camera.

Enzyme-linked immunosorbent assays

Ani-WNV antibodies were determined by ELISA, which was performed, with slight modifications, according to the methods described by Feinstein *et al.*[54] and Martin *et al.*[55] Briefly, Maxisorb 96-well plates were coated and incubated overnight at 4° with 100 μl of the different peptides (1 μg/well) or WNV antigen diluted 1 : 700 in coating buffer (0·1 M $NaHCO_3$, pH 9·6). After incubation, the coating buffer was decanted and the plates were washed four times in phosphate-buffered saline containing 0·05% Tween-20 (PBST). Each plate was blocked with 200 μl/well of 2·5% skim milk in PBST and incubated for 1 hr at 37° in a humidified atmosphere. Mouse serum samples were diluted (as indicated in the Results) in 2·5% skim milk in PBST and added (100 μl/well) to each well (two or three wells per sample) for 1 hr of incubation at 37° in a humidified atmosphere. Negative and positive control mouse sera were also tested in each plate. The plates were washed four times with PBST and 100 μl of 1 : 1000 diluted horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) was added to each well. After incubation at 37° in a humidified atmosphere for 1 hr, the plates were washed four times and 100 μl tetramethylbenzidine substrate was added to each well. The plates were then incubated at room temperature for 5–20 min, after which time the colour intensity was measured at an absorbance of 650 nm. For WNV-specific immunoglobulin isotype identification, WNV-antigen was used as above for coating, followed by blocking and application of serum at 1 : 1000 dilution. Then biotin-conjugated goat anti-mouse isotypes were applied followed by streptavidin-horseradish peroxidase.

Virus neutralization assay

The titre of viral neutralizing antibodies was determined using a modified plaque reduction neutralization test 50% ($PRNT_{50}$). Briefly, serial twofold dilutions (1 : 20 to 1 : 2560) of mouse sera were prepared in 96-well U-bottom plates. Then, $10^2 \times TCID_{50}$ of WNV (ISR98 strain) in equal volumes was added to duplicate wells of each dilution. After 1 hr of incubation at 4°, the serum–virus mixtures were added to a 96-well flat-bottom plate containing a monolayer of Vero cells. The plates were incubated for 4–5 days in a humidified atmosphere

at 37° and 5% CO_2 and then stained with crystal violet. Neutralizing antibody titres were expressed as the reciprocal of the highest dilution that gave 50% plaque reduction.

Preparation of spleen cell cultures and IFN-γ secretion

Spleens of immunized mice were harvested 10 days after the last immunization. The spleen pulp was extruded from the capsule into RPMI-1640 medium supplemented with 1% Pen-Strep, 10 mM HEPES, 2 mM L-glutamine and 5% FBS (RPMI-based medium). Cell suspensions were passed through a cell strainer, washed, treated for 2 min with ammonium chloride lysis solution (0·15 M NH_4Cl) for elimination of erythrocytes, and washed twice in RPMI-based medium. Splenocytes were resuspended in RPMI-1640 medium supplemented with 1% Pen-Strep, 10 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol and 10% FBS (RPMI-complete medium) and incubated in 96-well plates at a final concentration of 5×10^5 cells/100 μl/well. Splenocytes were stimulated *in vitro* with 25 μg/ml of p458 peptide, 5 μg/ml of the non-specific lymphocyte mitogen concanavalin A or they were not stimulated. The cells were then incubated at 37° in 5% CO_2 for 72 hr. The presence of IFN-γ in the various culture supernatants was determined by captured ELISA using commercial kits (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Results are expressed as absolute amounts as determined by comparison with an IFN-γ standard curve employed in the same assay.

Statistics

The significance of survival experiment results was calculated according to the Kaplan–Meier test.

The significance of the IFN-γ secretion results was calculated according to the single-factor (one-way) analysis of variance test.

Results

Immunization with the chimeric peptide p458-Ep15 protected mice from challenge with lethal doses of three different WNV strains

We first examined the protective efficacy of the p458-Ep15 chimeric peptide against three different WNV strains, namely the ISR98, NY99 and Goldblum strains. As a control to the p458 carrier effect we used the p431-Ep15 chimeric peptide where p458 was replaced by p431. Three-week-old mice were immunized as described in the Materials and methods. Seven days after the last injection, the mice were challenged with lethal doses of WNV and mortality was recorded for the following 21 days. As shown in Fig. 1, although survival of the control group (which received IFA only and was challenged with WNV-ISR98) was 0%, immunization with the chimeric p458-Ep15 peptide conferred a 100% protection against challenge with the same virus. No protection was achieved in the group of mice which were immunized with p431-Ep15 (0% survival), yet a slower mortality rate compared with the control group (IFA), was detected. In mice immunized with each of the peptides that compose the chimeric peptide (p458 or Ep15) alone, survival was approximately 25% and the mortality rate was slower compared with the control group. The 100% survival rate of the p458-Ep15-immunized group was significantly ($P < 10^{-6}$) greater than that achieved with the other peptides. The high protective efficacy of this chimeric peptide was also obtained when the immunized mice were challenged with the NY99 and the Goldblum strains, demonstrating survival rates of 90% and 95%, respectively (data not shown).

To investigate whether immunization with p458-Ep15 resulted in clearance of WNV, we examined levels of virus in the brain, the prime target organ of this neurotropic virus. Levels were determined by RT-PCR and plaque assays on day 7 after challenge (Fig. 2). Infectious viral

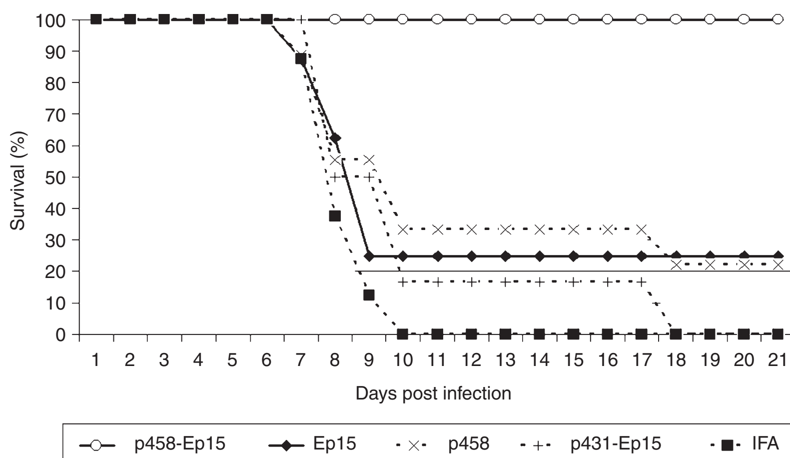

Figure 1. *In vivo* protective efficacy of p458-Ep15 chimeric peptide. Groups ($n = 6$ to $n = 9$) of 3-week-old mice were immunized three times with p458-Ep15, Ep15, p458 and p431-Ep15 peptides (equimolar amounts, emulsified in incomplete Freund's adjuvant (IFA), at 7-day intervals. Seven days after the last immunization, mice were challenged intraperitoneally with 10^4 plaque-forming units of WNV-ISR98 strain. Mortality was monitored for 21 days. For p458-Ep15 immunization $P < 10^{-6}$ compared with immunization with IFA or with p431-Ep15 and $P < 0.005$ compared with immunization with p458 or with Ep15 (Kaplan–Meier test).

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Treatment	Virus titres (PFU/0·1 g tissue)	RT-PCR WNV Ep	RT-PCR β-actin
IFA injection WNV-ISR98 challenge	1·4 x 10^8		
p458-Ep15 immunization WNV-ISR98 challenge	<10^1		
Control naïve	<10^1		

Figure 2. Viral loads following p458-Ep15 immunization and West Nile virus (WNV) -ISR98 challenge. Seven days after challenge, mice were killed and the brains were removed, also from control-naive mice (non-immunized and non-infected age- and sex-matched mice). Viral loads were assessed by reverse transcription–polymerase chain reaction (RT-PCR) for viral genome and by plaque assay for infectious virus titres [plaque-forming units (pfu)/0·1 g brain tissue]. The RT-PCR results represent results from one of six experimental mice, while infectious titres are the average of six brains.

titres reached $1·4 \times 10^8$ pfu/0·1 g brain tissue in control moribund mice (injected with IFA and challenged with ISR98), whereas in the control-naive (uninfected) and in the p458-Ep15-immunized and challenged group, no virus could be detected in any of the surviving mice (Fig. 2). Similarly, RT-PCR showed no indication of the presence of viral genome in control-naive or immunized and challenged groups (Fig. 2).

Antibody responses in mice following immunization with p458-Ep15

In view of the high degree of protection conferred on mice after immunization with the chimeric p458-Ep15 peptide, we further investigated the nature and specificity of the immune responses. We first tested the WNV-specific antibody response. Mice were immunized as described earlier. Seven days after the last injection, mice were bled and the sera were collected and pooled. Reactivity against WNV antigen was measured by ELISA. Immunization with p458, Ep15 and p431-Ep15 did not elicit detectable levels of WNV antigen-specific antibodies. This was in contrast to immunization with p458-Ep15, which induced high levels of WNV antigen-specific antibodies compared with immunization with other peptides (Fig. 3). As shown in Table 1, the titre of antigen-specific antibodies in p458-Ep15-immunized mice, 7 days after the last immunization was ≥ 25 600 and below the detection level after immunization with Ep15 or p431-Ep15 (Table 1). Next, we tested antibody levels in immunized and challenged mice 10 days after the challenge. Antibody titres in the p458-Ep15 group reached the high level of 51 200 whereas the antibody titres in mice immunized with Ep15-based formulations, Ep15 or p431-Ep15, were 3200 and 6400, respectively. The antibody titre in p458-immunized mice was 1600 (Table 1).

We further assessed the neutralizing efficacy of antibodies induced following p458-Ep15 immunization, with or without WNV challenge and of sera from p458-immunized mice with or without challenge. The WNV-neutralizing antibody titres were < 1 : 20 in naive and p458-immunized mice (Table 2). Immunization with p458-Ep15 induced a titre of 1 : 80 whereas following virus challenge, titre was enhanced by > 32-fold (1 : 2560). This titre was also higher (> 16-fold) than the neutralizing titre of p458-immunized and WNV-challenged mice (1 : 160). The observations therefore point to the contribution of pre-vaccination with the p458-Ep15 peptide to the development of an efficient response against subsequent WNV challenge. Evaluation of WNV-specific antibody isotypes in sera taken 6 months after vaccination and sub-lethal challenge also support the advantage induced by challenge-mediated enhancement of vaccination-induced immunity; antibody response (IgG1, IgG2a and IgG2b isotypes) to WNV antigen was considerably higher in vaccinated and challenged mice compared with mice challenged without vaccination (Table 3).

The p458 component of the p458-Ep15 formulation induces IFN-γ secretion

The level of cytokines released by T cells reflects the polarity and magnitude of the immune response. The

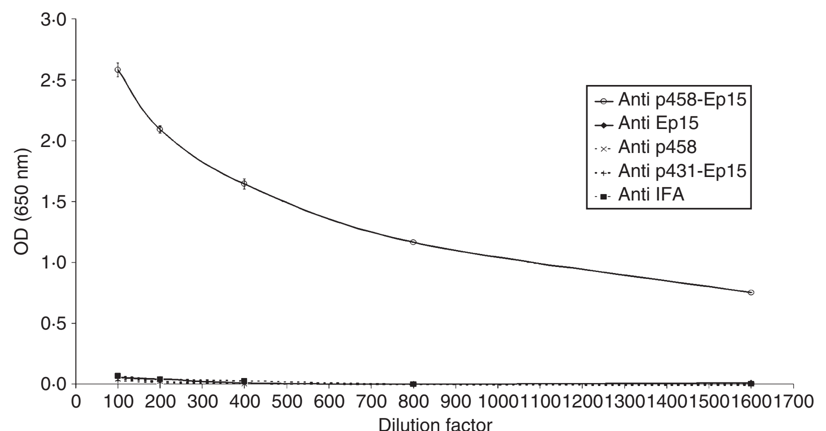

Figure 3. Levels of West Nile virus (WNV) -specific antibodies in sera of p458-Ep15-immunized mice. Groups ($n = 3$) of 3-week-old mice were immunized with the different peptides as described. Seven days after the last immunization, the mice were bled, sera from each group were pooled and tested by enzyme-linked immunosorbent assay. Results shown (bars) are representative of two independent sera titration experiments, ± SD.

Table 1. Titre of West Nile virus (WNV) antigen-specific antibodies in sera of immunized mice with or without challenge with WNV

Vaccination	WNV challenge	Date of sera harvest	Antibody titre
No treatment	–		Negative control[1]
p458	–	7 days after 3rd immunization	Negative control[1]
p458-Ep15	–	7 days after 3rd immunization	25 600
Ep15	–	7 days after 3rd immunization	Not detected[2]
p431-Ep15	–	7 days after 3rd immunization	Not detected[2]
p458-Ep15	10^4 PFU	10 days after challenge	51 200
Ep15	10^4 PFU	10 days after challenge	3200
p431-Ep15	10^4 PFU	10 days after challenge	6400
p458	10^4 PFU	10 days after challenge	1600

Three-week-old mice were immunized three times with the different peptides (equimolar amounts, emulsified in incomplete Freund's adjuvant), at 7-day intervals. Seven days after the last immunization, the mice were bled and then challenged intraperitoneally with a lethal dose of WNV-ISR98 strain. Ten days after the challenge, surviving mice were bled, sera from each group were pooled and tested by enzyme-linked immunosorbent assay as described in the Materials and methods.

[1]For calculation of cut-offs at a confidence level of 99·5%,[62] we employed two negative controls, namely pooled sera from (i) naive mice and (ii) p458-immunized mice.

[2]The detection level for WNV antigen-specific antibody titre in this experiment was 400.

PFU, plaque-forming units

Table 2. The presence of West Nile virus (WNV) -neutralizing antibodies in the sera of immunized mice following challenge with WNV

Vaccination	WNV challenge	Date of sera harvest	Neutralizing antibody titre
No treatment	–		< 1 : 20
p458	–	7 days after third immunization	< 1 : 20
p458-Ep15	–	7 days after third immunization	1 : 80
p458-Ep15	10^4 PFU	10 days after challenge	1 : 2560
p458	10^4 PFU	10 days after challenge	1 : 160

Three-week-old mice were immunized as previously described. Seven days after the last immunization, the mice were bled and challenged intraperitoneally with 10^4 plaque-forming units (PFU) of the WNV-ISR98 strain. Ten days after challenge, the mice were bled, sera from each group were pooled and tested for WNV neutralization capability, as measured by plaque reduction neutralization test 50% as described in the Materials and methods.

hsp 60-p458 peptide was reported to facilitate specific T-cell-mediated IFN-γ secretion.[36,38] To evaluate whether p458 used as a carrier for the B-cell epitope, Ep15, also induced IFN-γ secretion, we immunized mice with p458 or p458-Ep15. As shown in Fig. 4, *in vitro* stimulation of splenocytes from p458-immunized mice with p458 induced high levels of IFN-γ secretion. Indeed, secretion was specific because stimulation without p458 resulted in undetectable amounts of IFN-γ. Stimulation of splenocytes from p458-Ep15-immunized mice with p458 also induced high levels of IFN-γ secretion. As above, without *in vitro* stimulation with p458, IFN-γ levels were undetectable (Fig. 4). Pre-immunization with p458 or p458-Ep15 was imperative to IFN-γ secretion following *in vitro* stimulation with p458, because IFA-immunized mice did not induce significant levels of IFN-γ after *in vitro* stimulation with p458. Concanavalin A *in vitro* stimulation of splenocytes from all groups resulted in prominent IFN-γ secretion, emphasizing the specificity of the results observed following *in vitro* stimulation with p458 (Fig. 4).

Table 3. Characterization of the different immunoglobulin isotypes present in immunized mouse sera 6 month post challenge

Vaccination	WNV challenge	Isotype (OD, 1 : 1000 sera dilution)					
		IgA	IgM	IgG1	IgG2a	IgG2b	IgG3
No treatment	–	0·00	0·34	0·00	0·06	0·00	0·00
	Sub-lethal dose	0·03	0·46	1·14	1·11	0·86	0·12
p458-Ep15	–	0·00	0·46	0·00	0·10	0·00	0·00
	Sub-lethal dose	0·02	0·48	1·78	1·68	1·23	0·01

Three-week-old mice were immunized as previously described. Seven days after the last immunization, mice were inoculated intraperitoneally with a sub-lethal dose of West Nile virus (WNV; Goldblum strain). Six months after challenge, mice were bled, sera from each group were pooled and WNV-specific immunoglobulin isotype assay was performed by enzyme-linked immunosorbent assay as described in the Materials and methods. WNV-specific antibody response to immunized and challenged mice was significantly better compared with that of challenged mice [$P < 0·05$, analysis of variance one-tailed, pooled results for the main isotypes: immunoglobulin G1 (IgG1), IgG2a and IgG2b].

Discussion

The results reported here describe the protective efficacy and immunogenicity of a peptide-based chimeric vaccine against WNV. The chimeric peptide consisted of a B-cell epitope namely Ep15 and a T-cell carrier peptide p458-hsp 60. Several strategies to develop anti-WNV vaccines are currently being explored (reviewed in refs [12,17]), where the WNV-E envelope protein is the antigen of focus. We concentrated on the E-DIII domain because many neutralizing antibodies against WNV and other flaviviruses recognize epitopes within this domain.[29–31,34,56] We previously reported that peptide Ep15 derived from WNV E-DIII (amino acids 355–369) is a continuous B-cell epitope specifically recognized by antibodies from humans and

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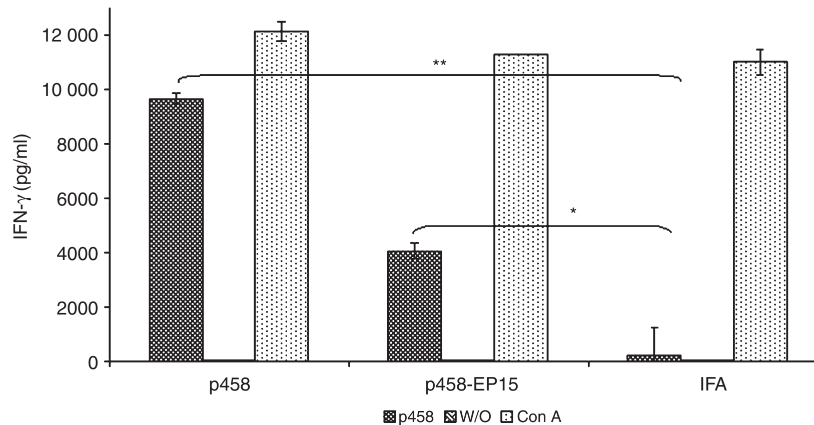

Figure 4. Interferon-γ (IFN-γ) secretion from splenocytes of immunized mice following *in vitro* stimulation with heat-shock protein 60 (hsp 60) -p458. Three week-old mice were immunized three times with p458-Ep15 and p458 as previously described. Seven days after the last immunization, mice were killed and spleens were harvested. Splenocytes were either non-stimulated, or stimulated with 25 μg/ml of p458. As a positive control, splenocytes were stimulated with 5 μg/ml concanavlain A (Con A). Splenocytes were then incubated for 3 days at 37°. IFN-γ secretion levels were measured by enzyme-linked immunosorbent assay. Results shown (bars) are representative of three independent experiments, ± SD. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

geese exposed to WNV infection.[32] To provide a link between innate and adaptive immunity and enhance the potency of the Ep15 peptide, we combined Ep15 with a T-cell carrier peptide, i.e. p458- hsp 60.[36–40] Healthy individuals or mice exhibit natural T-cell reactivity to several epitopes of the self-hsp 60 molecule. Among those, p458 was identified as a dominant epitope for such reactivity in BALB/c mice.[36,37] Indeed, it was previously shown that p458 in a conjugate vaccine provided T-cell help in an antibody response to the capsular polysaccharides of *Salmonella typhi*, *Streptococcus pneumoniae* type 4 and *Neisseria meningitidis* type B.[36–38,40] We also demonstrated that immunization with a chimeric peptide, p458 and a murine cytomegalovirus cytotoxic T lymphocyte epitope induced specific sustained IFN-γ secretion in the splenocytes and salivary gland from immunized mice, and enhanced clearance of murine cytomegalovirus from salivary gland.[39]

In view of these data, we constructed the synthetic linear chimeric peptides, p458-Ep15, in which the hsp 60-p458 sequence was synthesized, in a linear manner, with the Ep15 sequence. The results of the *in vivo* studies showed unequivocally, that the formulation based on the linear chimeric peptide containing both p458 and Ep15 components conferred a significant degree of protection against challenge with three different virulent strains of WNV. This protection was greater than achieved with the Ep15-based formulations that did not contain p458, i.e. p431-Ep15. In addition, p458-Ep15-induced protection was significantly better than that obtained with Ep15 alone (Fig. 1). Complete WNV clearance from the brain was observed (Fig. 2). To achieve the highest degree of protection, mice had to be immunized three times, with 1 week apart between each injection; using two immunizations conferred a lesser degree of protection (data not shown). The benefit of immunization with the p458-Ep15 chimeric peptide can be attributed to the T-cell help induced by the p458 component, as we have shown before for other disease models.[36–40] In particular, the higher efficacy of the Ep15-p458 could be attributed, in part, to p458-mediated IFN-γ secretion (Fig. 4). Another explanation to the contribution of p458 to the increased protective efficacy of the chimeric peptide is its ability to trigger innate immunity. The hsp 60 is an endogenous ligand for TLR4 expressed by antigen-presenting cells as macrophages and dendritic cells.[42] The hsp 60-p458 peptide bears some TLR4-binding characteristics of the intact hsp 60 protein.[43] Therefore, the p458 component of the p458-Ep15 can serve as a stimulus for TLR4 expressed by antigen-presenting cells. The use of p458 as an innate adjuvant is in accordance with modern approaches for vaccine development that involve agonists to innate immune receptors, particularly to TLR4. Yet, typically these agonists are non-self. Common to either self or foreign TLR4 agonists is the induction of potential inflammatory reactions elicited via TLR4 including fever because of IFN-γ-activated macrophages making pro-inflammatory cytokines. Potential risk of complications should be considered. The prominence of hsp 60-p458 as a carrier peptide, compared with the universal tetanus toxoid and its derived peptides,[37–39] clearly points towards an innate immunity component of the hsp 60-p458 carrier peptide in addition to its adaptive component serving as an major histocompatibility complex Class II-restricted natural self-epitope for T helper cells.[36,37]

An important question asks whether immunization with self-peptide p458 might induce an autoimmune disease. Data from previous publications show clearly that the use of hsp 60 and/or fragments thereof (e.g. p458) does not induce autoimmune disease.[36–38,57–59] Nonetheless, the question of autoimmunity still remains to be unequivocally proven. However, it is encouraging to note that humans are born with natural autoimmunity to hsp 60 epitopes;[60] this is compatible with the notion that autoimmunity to hsp 60 peptides is not likely to cause disease. Indeed, a peptide epitope of hsp 60 partially overlapping with p458 is currently in advanced clinical trials for the arrest of autoimmune diabetes.[61] In any case, large numbers of mice have been vaccinated with a variety of p458 conjugates and no autoimmune disease has yet been observed.

The Ep15 sequence (amino acids 355–369) is nearly identical to different reported sequences of WNV strains, except for a V364M change in the CN99 strain (mosquito), an A365S change in the AUS60 WNV strain and a S369A change in several other WNV strains.[7,30] In this study, we used the LVTVNPFVSVATANS sequence, reported in the original 1937 human isolate from Uganda (accession no. AAA48498). In the three strains we studied, the Goldblum-Ep15 peptide was identical to the Ep15 sequence of the original Uganda isolate, whereas ISR98-Ep15 and NY99-Ep15 differed at position 369 (i.e. S369A). Evidence that Ep15 is recognized by neutralizing anti-WNV antibodies has already been presented: (i) A369 was suggested to play a role in the determination of antigenic specificities,[33] and (ii) residues A365 to N368 were reported to be contacted by a WNV-neutralizing antibody.[34,35] Despite the difference in position 369, the p458-Ep15 formulation conferred significant protection against the ISR98 (Fig. 1), NY99 strain and the Goldblum strain (data not shown). In the protected mice, we were unable to demonstrate virus spread and replication in the brain, indicating that after challenge an effective anti-WNV immune response has taken place.

Immunization with p458-Ep15 has induced high levels of antibodies specific to Goldblum-derived WNV-antigen. These antibodies were also able to neutralize infection with the WNV-ISR98 strain used in the neutralization assays (Table 2). These results demonstrate the capacity of Ep15 to induce a WNV-specific antibody response against a variety of WNV strains, in spite of the S369A mutation. Interestingly, challenge of p458-Ep15-immunized mice with WNV enhanced the WNV-specific antibody titre twofold in the ELISA (Table 1), and the neutralizing antibody titre by 32-fold (Table 2). These data suggest that immunization with p458-Ep15 elicits a primary specific immune response which is later enhanced to a secondary response following the WNV challenge. This argument is supported by the results of neutralizing antibody assays. The differences in the titre after challenge with WNV, was fourfold higher in the p458-Ep15-immunized mice than in mice that were immunized with p458 and had no prior WNV specific antibody. Studying WNV-specific antibodies in sera taken 6 months after vaccination and sub-lethal challenge also support the advantage induced by challenge-mediated enhancement of vaccination-induced immunity; antibody response to WNV antigen was considerably higher in vaccinated and challenged mice compared with mice challenged without vaccination (Table 3). The results above also point out the adjuvant effect of p458; the levels generated with Ep15 alone after WNV challenge were lower than those obtained with p458-Ep15.

The results presented here provide good evidence of the potential of the chimeric peptide p458-Ep15 to be used in a vaccine formulation against WNV. A non-infectious, peptide vaccine can be beneficial for use in individuals with a weak immune response, such as the elderly, where the administration of a live virus vaccine can have adverse effects. This vaccine can provide the first line of defence, which will provide the initial immunity that can later be enhanced by live virus vaccine or by herd immunity.

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