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West Nile virus is neutralized by HOCl-modified human serum albumin that binds to domain III of the viral envelope protein E

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Abstract

The outbreaks of West Nile virus (WNV), an emerging flavivirus recently implicated in outbreaks of fatal encephalitis, necessitate the development of effective anti-WNV drugs. In this study, it is demonstrated that human serum albumin is transformed into a WNV antiviral substance by hypochlorite (HOCl) modification. The HOCl-modified albumin efficiently neutralized WNV in vitro (EC_{50}=300 nM) and showed binding to a recombinant protein, representing the domain III of the WNV external envelope E glycoprotein.

Keywords: West Nile virus; Domain III; Neutralization; HOCl-modified proteins

Introduction

West Nile virus (WNV) belongs to the family *Flaviviridae*, a large virus family that comprises more than 70 different viruses. There is no specific treatment, drug or a vaccine available to cure or prevent humans of WNV infection. In the recent years, WNV outbreaks had been observed with significant rates of mortality in humans (Vorou et al., 2007; CDC, 2007) and in combination with its geographical expansion there is a pressing need to develop WNV treatment options.

West Nile virions are spherical in shape (40–60 nm) and are covered by a lipid envelope containing envelope proteins E and M. In patients with WNV infection, neutralizing antibodies can be detected (Niedrig et al., 2007; Planitzer et al., 2007), which are preferentially directed against the E protein (Oliphant et al., 2005). The E protein is divided into three domains where antibodies binding to the domain III potently neutralize WNV in vitro and in vivo (Oliphant and Diamond, 2007; Throsby et al., 2007) in contrast to antibodies binding to other domains in the E protein (Throsby et al., 2006). Therefore, a recombinant protein representing domain III was principally used to detect the antibody response in serum samples of WNV-infected individuals directed against domain III (Ludolfs et al., 2007). Since domain III is a target for WNV-neutralizing antibodies and since a peptide, representing the domain III sequence, can inhibit viral infection (Chu et al., 2005), it is conceivable that this region plays an important role in the entry process of WNV. These data suggest that blocking domain III by a domain III-binding product would efficiently inhibit viral infection.

Besides the specific humoral immune response, other non-specific immune responses play a role in the defence against pathogens. Such a non-specific immune response is provided by neutrophils and eosinophils. Both cell types are packed with peroxidases, with myeloperoxidase (MPO) being the most abundant protein in the granules of neutrophils. Myeloperoxidase is the central enzyme of the so-called MPO/H_2O_2/halide system (Harrison and Schultz, 1976). The major product of the MPO/H_2O_2/halide system is hypochlorite, which is generated by H_2O_2 oxidation of chloride. When neutrophils get activated, for example by a soluble stimulus such as phorbol esters, formylated peptides or cytokines, they undergo a process referred to as a respiratory burst. During this process, the content of the granules is released into phagolysosomes, but is also released into the medium outside the cell. As a result, HOCl is present in the cell-

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free environment able to kill pathogens but also able to modify serum components.

In the past, it has been thought that HOCl acts directly against pathogens based on its strong oxidative power (Klebanoff and Coombs, 1992). In recent years, HOCl-modified components were identified in blood and tissue (Marsche et al., 2007; Hammer et al., 2001) using monoclonal antibodies directed against HOCl-induced conformational changes (Gröne et al., 2002; Malle et al., 1995) or directed against HOCl-induced modifications of amino acid residues (Gujral et al., 2004; Shao et al., 2005).

Although the MPO enzyme as well as the chemical process by which MPO is producing HOCl is very well characterized (Klebanoff, 2005), the exact mechanism how microbial killing occurs is still controversial. HOCl is thought to act directly against pathogens as a toxic bleach. On the other hand, it is conceivable that HOCl-modified components play an active part in microbial inactivation.

Here in this study, HOCl-modified human serum albumin (HSA) was generated and tested for its ability to neutralize WNV in cell culture. We observed a potent inhibitory effect on WNV infection (EC_{50} = 300 nM), whereas infection of cells by Dengue-2 (DEN-2) and Yellow fever (YF) viruses remained unchanged. Surface plasmon resonance (SPR) analysis demonstrated that HOCl-modified HSA bound specifically to the WNV envelope E domain III, which was tested in form of a recombinant protein. Thus, neutralization of WNV was clearly correlated with binding of the inhibitor to the envelope E domain III.

Results and discussion

By using 6000 $TCID_{50}$ of WNV we observed, that after 3 days more than 98% of the Vero B4 cells were infected (Fig. 1A). This infection rate was reduced to 53% by mHSA at 10 μg/ml; to 47% by 20 μg/ml and to 30% by 40 μg/ml. No infected cells were detected at 80 μg mHSA/ml. Thus, WNV infection could be blocked by 50% with about 20 μg/ml of mHSA and a concentration of 80 μg mHSA/ml totally blocked infection. In these experiments, infection was totally blocked when mHSA was added to the cells together with virus supernatants. In the next

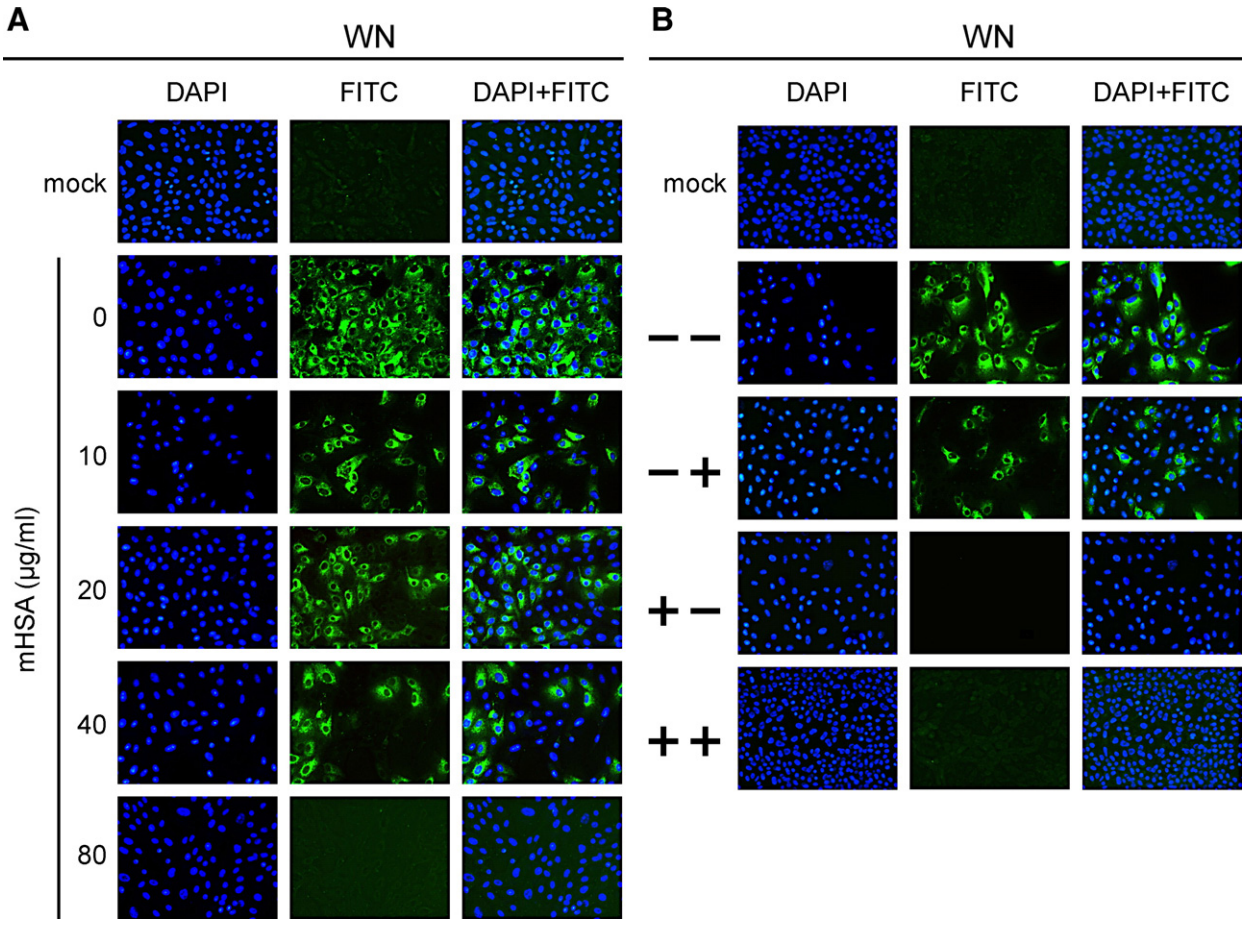

Fig. 1. Neutralization of West Nile virus by mHSA. Cells were inoculated with virus supernatants for 1 h. After 1 h, the virus supernatant was replaced against cell culture medium. Infection of cells was monitored by a protein E-specific monoclonal antibody 3 days post infection. The cellular DNA was stained by DAPI (4′,6-diamidino-2-phenylindol). (A) Vero B4 cells were infected with 6000 $TCID_{50}$ of WNV. The mHSA protein was present at time of infection and 1 h post infection until day 3 at various concentrations (0, 10, 20, 40, and 80 μg/ml). (B) (− −): Vero B4 cells infected with WNV supernatant containing 1000 $TCID_{50}$. No mHSA was added at time of infection and no mHSA was added 1 h post infection. (− +): Cells were infected without mHSA for 1 h. After 1 h, the cell culture medium was replaced and mHSA (80 μg/ml) was added to the infected cells. (+ −): Cells were incubated with WNV in the presence of mHSA (80 μg/ml). After 1 h the culture medium was replaced and no mHSA was added to the infected cells. (+ +): Cells were infected and mHSA (80 μg/ml) was present from time of infection until day 3.

experiment, we analyzed the effect of mHSA on viral spreading. Vero B4 cells were inoculated with WNV supernatants for 1 h, and after this period, cells were washed and the culture medium was replaced. Thus, the effect of mHSA could now be tested in a cell culture containing productively infected cells. As documented in Fig. 1B, a WNV dose representing 1000 $TCID_{50}$ caused an infection rate, on day 3, of about 63% (Fig. 1B; no mHSA present; − −). When WNV was given to the cells for 1 h and the medium was replaced by new medium containing 80 μg/ml mHSA, the rate of infection was reduced to 22% (Fig. 1B; − +). No infected cells were observed when mHSA was present at the time of infection (Fig. 1B; + +;+ −). The experiments document that viral spreading in cell cultures, which were previously infected by WNV, was efficiently blocked by mHSA (Fig. 1B; − +).

Next we have investigated the effect of mHSA on cell proliferation and cell death (Fig. 2). In both assays, no negative effect could be observed for concentrations that were 10 times higher than the 50% inhibitory dose of about 20 μg mHSA/ml. As an additional control, we studied the viral growth of YF and DEN-2 viruses in Vero B4 cells in the presence of mHSA. Firstly, we studied viral entry by identification of virus antigen in an immunofluorescence assay. Up to a final concentration of 160 μg mHSA/ml we observed normal infection rates for DEN-2 and YF viruses (Fig. 3A). Secondly, we have tested viral growth of WNV, DEN-2 and YF viruses, in the presence of 100 μg/ml mHSA, over a period of 9 days (Fig. 3B). Again, viral replication of DEN-2 and YF was not reduced in the presence of mHSA, whereas WNV replication was completely blocked. Thus, mHSA was specific against WNV and no unspecific toxic side effect against Vero B4 cells, YF or DEN-2 viruses could be observed. Since the viral growth of YF and DEN-2 was not inhibited, it is suggested that mHSA will not inhibit the cellular metabolism or any other intracellular mechanism needed to produce these viruses.

The experiments suggest that WNV infection is blocked at the entry level. For WNV, the domain III of the E protein is important for viral entry and since monoclonal antibodies directed against this domain can block infection efficiently, we have investigated the role of mHSA in binding to domain III. To generate domain III, we have cloned its 101 amino acid coding sequence into *Escherichia coli* expression vector pET22b and expressed and purified it as a 6× His-tagged protein as documented in Fig. 4. To study domain III–mHSA interaction, we used the method of surface plasmon resonance (SPR) spectroscopy. With this method, binding of a protein in solution to an immobilized protein on a biosensor can be studied in real time. We investigated domain III-to-mHSA binding in two ways. Firstly, mHSA was immobilized on a biosensor and domain III was studied for binding. Using this experimental design, we observed strong binding of WNV domain III (Fig. 5A) and no binding of WNV domain III to a HSA coated biosensor (Fig. 5B) or to the biosensor itself (data not shown). In addition to WNV domain III we also tested recombinant domain III proteins of DEN-2 and YF viruses for binding to mHSA. As documented in Fig. 5E, the binding of DEN-2 and YF domain III was significantly lower compared to the binding of WNV domain III to mHSA. A comparison of the RU values for WNV domain III binding (Fig. 5E) also showed that binding to immobilized

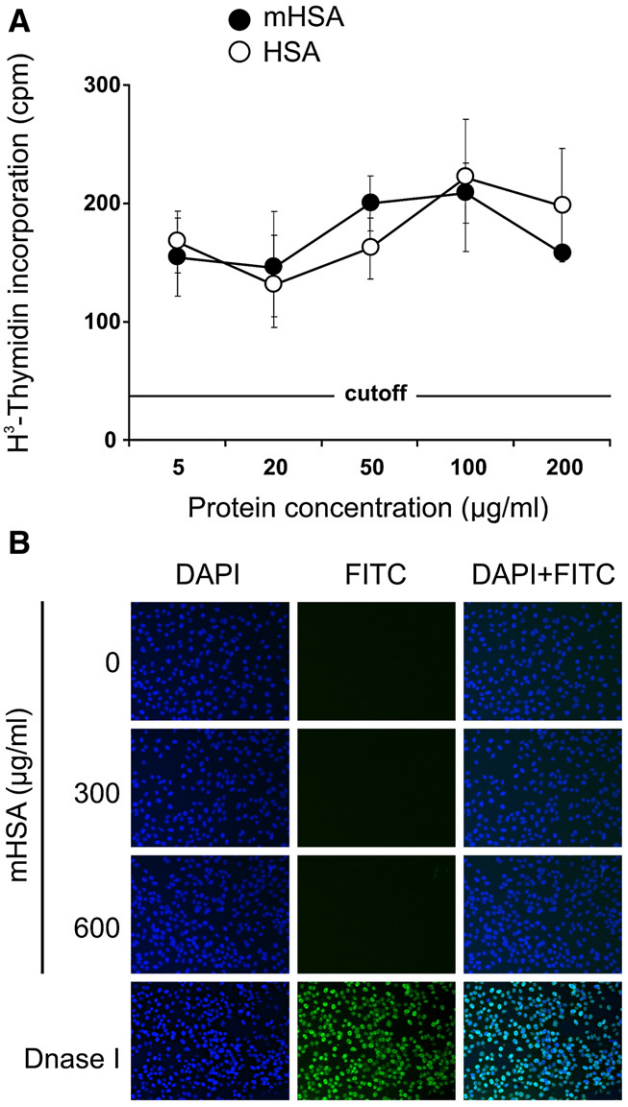

Fig. 2. Effects of mHSA on cell proliferation and cell death. (A) ^{3}H-Thymidine incorporation assay. Vero B4 cells were cultured in 96-well plates in the presence of mHSA (black) or HSA (white) at concentrations of 5, 20, 50, 100, and 200 μg/ml. Each test was performed in triplicate. (B) TUNEL assay. Vero B4 cells were cultured on 8-well chamber slides in the presence of 0, 300 and 600 μg/ml mHSA. TdT labeling with fluorescein-dUTP was performed on day 3. Cells were treated with DNase I as a positive control.

mHSA was concentration dependent. Secondly, we have immobilized domain III onto a biosensor and have now investigated the binding of mHSA (Fig. 5C). In accordance with the first SPR results, we observed again strong binding of mHSA to WNV domain III. No binding of HSA to the immobilized WNV domain III was observed (Fig. 5D). A comparison of the results of the second experimental design (Fig. 5F) showed that mHSA binding to immobilized WNV domain III was also specific as well as concentration dependent. In the SPR study, the interaction between mHSA and the domain III of WNV was clearly demonstrated, suggesting that mHSA inhibits infection by binding to the WNV envelope E protein.

The modification of HSA by HOCl is a very complex chemical reaction and data on the modifications induced by HOCl are

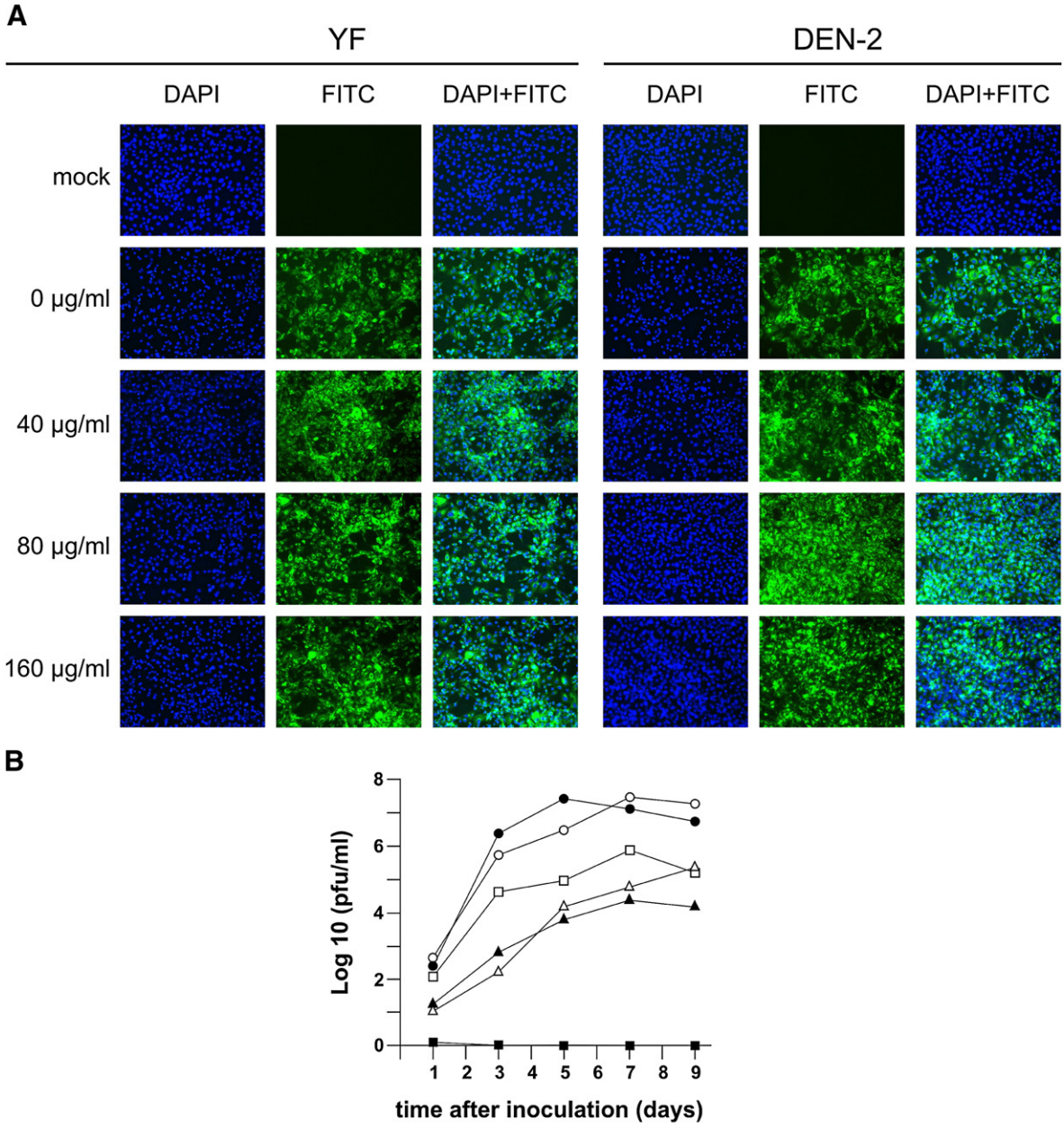

Fig. 3. Effects of mHSA in YF and DEN-2 virus cultures. (A) Vero B4 cells were inoculated with (i) cell culture supernatants containing 1000 $TCID_{50}$ of YF and DEN-2 virus and (ii) mHSA at various concentrations. After 1 h, the virus containing culture medium was replaced against medium containing the appropriate concentration of mHSA. Virus-infected cells were detected on day 3 using a protein E-specific antibody. Cells, i.e. the cellular DNA, were stained by DAPI. (B) Replication kinetics of WNV, DEN-2 and YF virus in the presence (black) and absence (white) of 100 µg/ml mHSA. Cells were infected at a multiplicity of infection of 0.1 with WNV (squares), DEN-2 (triangles), YF (circles). After 1 h, the medium was replaced against medium containing the appropriate mHSA concentration. Virus titers were measured by a plaque assay on days 1, 3, 5, 7 and 9.

available only in the context of single amino acids or small linear peptides (Hawkins et al., 2003). Various studies on protein HOCl oxidation have shown that side-chain modification, cross-linking as well as fragmentation can be observed. However, Cys and Met amino acids react most rapidly with HOCl (Pattison and Davies, 2001), which can lead to cross-linking by the formation of intra- and intermolecular sulfonamides (Fu et al., 2002). Another known reaction of HOCl with proteins is the formation of 3-chlorotyrosine and 3,5-dichlorotyrosine. Both chlorotyrosine species are used as markers for HOCl- or MPO-induced reactions in vitro and in vivo (Kettle, 1996; Hazen and Heinecke, 1997). In a recently published study by Noueiry et al. (2007), a secondary sulfonamid (AP30451) containing a 1-(3-chloro-4-methylphenyl) group was identified as a new class of small molecule inhibitors of WNV. We do not argue that HOCl-modified serum albumins are a molecular mimicry of these small molecular inhibitors, but HOCl treatment of serum albumins seems to produce new functional groups which cause protein binding activity for the WNV domain III and the chemical modifications might be more specifically and not that randomized

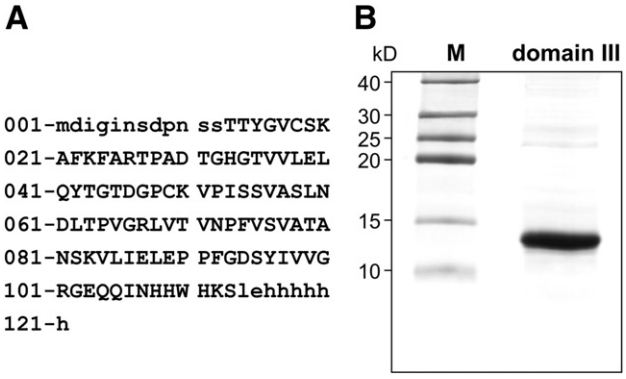

Fig. 4. Expression and purification of the West Nile domain III. (A) Amino acid sequence of the WNV domain III. Small letters, amino acids encoded by the expression plasmid pET22b. (B) Polyacrylamide gel electrophoresis of His-tag affinity-purified domain III protein.

as was thought in the past (Salavej et al., 2006; Temple et al., 2006). Nonetheless, this study provides evidence for the hypothesis that HOCl-induced oxidative changes in host proteins can play an important role in the host defence against pathogens.

Materials and methods

Chemical modification of HSA

Human serum albumin was treated with hypochlorous acid (HOCl) as described by Malle et al. (2000). In brief, HSA (Sigma-Aldrich) was diluted in PBS pH 7.5 and HOCl was added for 15 min at 4 °C. The molar ratio of HSA/HOCl was 1:500 and the HOCl concentration was controlled by UV spectroscopy ($\varepsilon = 375$ mol/cm l). After incubation, the remaining HOCl was separated by gel filtration (Sephadex G10) and concentrated by ultrafiltration (MWCO 10.000 D, Millipore) to a final concentration of 10 mg/ml. The HSA protein treated with a 500-fold molar surplus of HOCl was designated mHSA (modified-HSA).

Effects of mHSA and HSA on Vero B4 cell proliferation

^{3}H-Thymidine incorporation assay: Vero B4 cells (DSMZ ACC 33, African green monkey kidney) were cultured in the presence of mHSA or HSA at concentrations of 5, 20, 50, 100, and 200 μg/ml. Vero B4 cells were seeded at 10^4 cells per well in 96-well plates. Each test was performed in triplicate. On day 2, 0.2 μCi ^{3}H-thymidine was added to each well. After 8 h, the DNA was harvested, bound to a glass fibre membrane and incorporated ^{3}H-thymidine was monitored using a β-counter.

Direct TUNEL (terminal transferase dUTP nick end labeling) assay: For the detection of mHSA-induced cell death, we used a commercial kit (Roche Applied Science, USA) and performed the assay according to the manufacturer's recommendations. Strand breaks in cellular DNA present in apoptotic cells were labeled with fluorescein-dUTP by the enzyme TdT (terminal deoxynucleotidyl transferase). As a control, vital as well as DNAse-treated Vero B4 cells were tested to determine the background of apoptotic cells under normal cell culture conditions.

Neutralization of WNV by mHSA

Vero B4 cells were infected with WNV (strain Wengler, SwissProt P06935, GenBank Accession Number M12294) and infection was monitored by IFA using a glycoprotein E-specific antibody. Cells were incubated with virus containing supernatants (6000 $TCID_{50}$) for 1 h to allow infection. After this period, the cell culture medium was replaced. The neutralization experiments were different in the way mHSA was added. Modified HSA was added to the cell cultures together with virus supernatants or mHSA was added 1 h post infection. The infection of Vero B4 cells was monitored 3 days post infection by IF staining using the glycoprotein E-specific mAb 15R4 (Ludolfs et al., 2002) and a second anti-mouse FITC-conjugated antibody (Sigma-Aldrich, USA). The experiments were carried out in 8-well glass slides (chamber slide system; Nalge Nunc Int., USA).

As a control, Vero cells were infected with Yellow fever virus (strain 17D; Theiler and Smith, 1937) or Dengue-2 Virus (strain New Guinea C, GenBank Accession Number AF038403). Viral infection by YF and Den-2 was detected 3 days post infection using the YF protein E-specific mAb AK6330 (from Niedrig M., Robert Koch Institute, Berlin, Germany) or a DEN-2 protein E antibody-positive human serum sample (from Schmitz H.; Bernhard Nocht Institute, Hamburg).

Plaque assay

Serial dilutions of cell culture supernatants starting at 10^{-3} were given to Vero B4 cells grown on 6-well plates. After 1 h of incubation, cells were washed and overlaid with 3 ml of medium containing 0.5% agarose. After 7 days, 2 ml of 7% formaldehyde was added for 30 min. Overlays were removed and the cell monolayer was stained using 0.5% crystal violet.

Expression and purification of the WNV domain III

The WNV domain III protein was generated as described earlier (Ludolfs et al., 2007). In brief, domain III was cloned into the *E. coli* expression vector pET22b and purified as a His-tagged protein from *E. coli* lysates by Ni-nitrilotriaceticacid (NTA) affinity chromatography in the presence of 7 M urea. The 7 M urea solution containing the WNV domain III protein was loaded onto a second Ni-NTA column. Bound protein was stepwise equilibrated until the buffer concentration was 100 mM Tris pH 7.5. Bound protein was eluted with Tris buffer (100 mM, pH 7.5) containing imidazole (1 M). The eluted protein was directly dialyzed against a PBS/20% glycerol solution. The final concentration of purified WNV domain III protein was 200 μg/ml.

Binding of mHSA to WNV domain III

Binding of mHSA to the domain III protein was monitored by surface plasmon resonance (SPR; Biacore) spectroscopy. Human serum albumin was immobilized on a dextran-coated, CH-activated C5 sensor chip (Biacore, Sweden) by NHS (*N*-hydroxysuccinimide)/EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) coupling at densities yielding

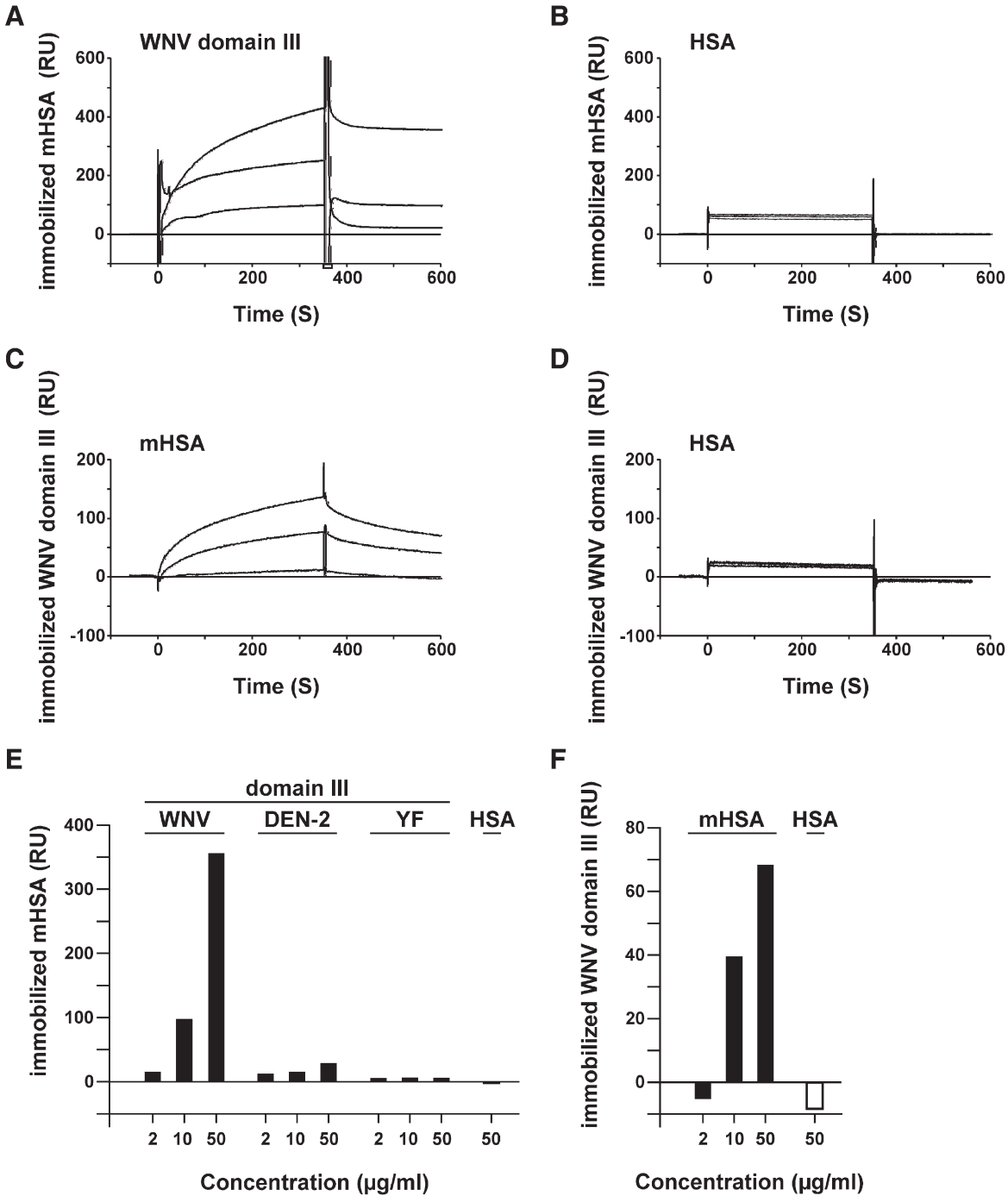

Fig. 5. mHSA binding to WNV domain III. Binding of domain III to immobilized mHSA (A, B, E) and binding of mHSA to immobilized domain III (C, D, F) was monitored by surface plasmon resonance (SPR; Biacore) spectroscopy. Domain III and mHSA were immobilized on a sensor chip (C5, Biacore, Sweden) at densities of 3900 or 3500 RU, respectively. Proteins were applied to the loaded sensor chips at a flow rates of 5 µl/min. Sensor grams shown in A to D represent RU differences obtained from measurements of the mHSA or domain III loaded sensor and a non-loaded reference surface. (A) Overlay of sensor grams showing binding of domain III protein to immobilized mHSA. Domain III was diluted in PBS/2 M urea and tested at concentrations of 50, 10 and 2 µg/ml (from top to bottom). (B) Same as in A, but overlay of sensor grams showing HSA binding to immobilized mHSA. HSA concentrations: 2, 10, 50 and 250 µg/ml in PBS. (C) Overlay of sensor grams showing binding of mHSA to immobilized domain III protein. mHSA was tested at concentrations of 50, 10, 2 µg/ml PBS (from top to bottom). (D) Same as in C, but overlay of sensor grams showing HSA binding to immobilized domain III protein. HSA concentrations: 2, 10, 50 and 250 µg/ml PBS. (E) Responses of WNV, DEN-2, YF domain III and HSA tested against immobilized mHSA at the end of the association phase (600 s), domain III binding (black), HSA binding (white). (F) Responses of mHSA and HSA tested against immobilized WNV domain III protein at the end of the association phase (600 s) as shown in C and D, mHSA binding (black), HSA binding (white).

3500 response units (RUs). Sensor chips loaded with mHSA were generated by treatment of a HSA-loaded sensor chip with a PBS/HOCl solution (10 mM HOCl) for 10 min at RT. The concentration of HOCl was monitored by UV spectroscopy ($\varepsilon = 375$ mol/cm l). The WNV domain III protein was immobilized by NHS/EDC coupling on a C5 sensor chip at densities yielding 3900 RUs. HSA and mHSA, diluted in PBS or domain III proteins diluted in PBS/2 M urea, were applied to the loaded sensor at a flow rate of 5 µl/min. Surface plasmon resonance spectroscopy was carried out using a Biacore 1000. After each test, the loaded sensor was purified using 500 µl of HCl (100 mM) at a flow rate of 100 µl/min.

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