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Characterization of Glycoprotein E C-End of West Nile Virus and Evaluation of Its Interaction Force with αVβ3 Integrin as Putative Cellular Receptor

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Abstract—Recombinant polypeptide containing the 260-466 amino acid sequence of West Nile virus (WNV) strain LEIV-Vlg99-27889-human glycoprotein E (gpE, $E_{260-466}$) was constructed. Immunochemical similarity between the $E_{260-466}$ and gpE of WNV was proven by enzyme immunoassay (EIA), immunoblot, competitive EIA, hemagglutination inhibition, and neutralization tests using polyclonal and monoclonal antibodies against the viral gpE and recombinant $E_{260-466}$. Polypeptide $E_{260-466}$ induced formation of virus neutralizing and cross-reactive antibodies that were interactive with various epitopes of this recombinant protein. It is shown by evaluation of the interaction of $E_{260-466}$ with one of the proposed cell receptors of WNV that average $E_{260-466}$–αVβ3 integrin-specific interaction force measured using atomic force spectroscopy was 80 and 140 pN for single and double interactions, correspondingly. Taken together with previously described interaction between laminin-binding protein (LBP) and WNV gpE domain II, it is proposed that WNV gpE can interact specifically with two cellular proteins (LBP and αVβ3 integrin) during virus entry.

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Key words: West Nile virus, glycoprotein E, atomic force spectroscopy, αVβ3 integrin

West Nile virus (WNV) belongs to the Japanese encephalitis virus (JEV) antigen group of the genus *Flavivirus*, family Flaviviridae, and causes West Nile fever (WNF) in humans [1]. WNV infection occurs in various forms in humans, from asymptomatic infection and mild fever to severe forms of encephalitis and meningoencephalitis with a mortality rate of 12-14% [2]. The WNV modern genotype Ia appeared in the Mediterranean area in the mid-1990s [3]. This WNV genotype extended across Africa, Europe, Asia, and South and North America in about 15 years. Rapid distribution of WNV through new geographic regions is connected with its unique ability to infect many species of mosquitoes, birds, and mammals [4]. One of the hypotheses explaining the ability of WNV to infect so many different species is the capacity of WNV virions to penetrate into cells of various species by receptor endocytosis through interaction with highly conservative cell receptor(s).

The WNV virion consists of a nucleocapsid containing single-stranded positive RNA and C protein, and a lipid envelope with E and M proteins embedded into it. Protein E mediates flaviviral attachment to cell receptors and determines the tropism, virulence, and production of virus-neutralizing antibodies [5]. It forms homodimers located parallel to the lipid envelope on the external surface of the viral particles [6]. The X-ray model of glyco-

Abbreviations: AFM, atomic force microscopy; EIA, enzyme immunoassay; gpE, glycoprotein E; LBP, laminin-binding protein; MAb, monoclonal antibodies; JEV, Japanese encephalitis virus; RMSD, root mean square deviation; TBEV, tick-borne encephalitis virus; WNF, West Nile fever; WNV, West Nile virus.

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protein E (gpE) includes an ectodomain formed by 1-395 amino acid residues (a.a.) and two transmembrane segments localized within the 396-496 a.a. of the C-end part of gpE. The ectodomain consists of three domains. Domain I (central) of the protein includes 1-51, 137-189, and 285-302 a.a. Domain II (dimerization domain) is also formed by different parts of the polypeptide chain (52-136 and 190-284 a.a.). It contains the highly conservative amino acid sequence of 98-110 a.a., that forms the cd-loop (fusion peptide), thus providing for virus–host cell membranes fusion in cell endosomes under low pH conditions [7]. It was predicted in our recent work that the bc-loop (73-89 a.a.) of domain II interacts with laminin-binding protein (LBP) and, together with the cd-loop (fusion peptide), determines initial stages of flaviviral penetration into the cell [8]. The existence of a proposed LBP–WNV domain II specific interaction was proven by both immunochemical and single molecule force spectroscopy methods: LBP interacted with WNV gpE in enzyme immunoassay (EIA) and immunoblotting, and the average values of the specific interaction force of 105 ± 20 pN (single interaction) and 210 ± 50 pN (double interaction) were determined for the LBP–WNV gpE domain II pair [9].

Analysis of X-ray data showed that domain III (303-395 a.a.) has Ig-like form [6]. The presence of the RGD sequence in this domain (388-390 a.a. for JEV) is indicative of its interaction with the receptors on the cell surface [10]. The recombinant WNV domain III blocks the penetration of WNV into Vero and mosquito C6/36 cells [11]. Domain III epitopes, which are purportedly able to interact efficiently with the cell receptor, were mapped by escape-mutants assay: X-ray analysis showed that the residues 307, 330, and 332 of this domain are located on the external surface of gpE [12, 13]. An important role of the flaviviral gpE domain III in virus-neutralizing antibodies production was confirmed by monoclonal antibody (MAb) panel investigations [14]. Flaviviral virulence and tropism depend on amino acid substitution in domain III [15]. The therapeutic antibodies E16 recognize an epitope within WNV gpE domain III [16].

Correspondingly, the lateral surface of domain III was predicted to be involved in receptor recognition. Besides other confirmations, this has recently been tested by atomic force microscopy (AFM) [17, 18]. It was shown that for a nominal force loading rate around 12 nN/sec, the force of single molecule specific binding between WNV gpE, domain III, and αVβ3 integrin is 45 ± 5 pN. At the same time, the authors also observed more infrequent interactions with force 83 and 124 pN that suggests the appearance of interactions between several molecule pairs under the chosen experimental conditions.

The gpE membrane anchor is formed by two of the membrane α-helices (401-413 and 431-449 a.a.) separated by the conservative sequence 414-430 a.a. There are two transmembrane segments: 450-471 and 473-496 a.a. The 450-472 and 431-449 a.a. regions are important for the stabilization of the gpE–protein M interaction during viral assembling and gpE trimerization under acidic pH conditions with the participation of 401-413 a.a. [19].

The purposes of the current study are (i) to analyze the immunochemical properties of the recombinant polypeptide mimicking the epitopes of the gpE C-end of the WNV Russian strain LEIV-Vlg99-27889-human, (ii) to create a collection of MAbs against this polypeptide and analyze their biological activity, (iii) to map functionally important epitopes within the gpE C-end, and (iv) to measure the force of single-molecule specific binding between the WNV gpE C-end and αVβ3 integrin.

MATERIALS AND METHODS

Virus and cell cultures. Experiments were performed with the WNV strain LEIV-Vlg99-27889-human (Vlg 27889) (originally isolated from the brain of a patient from Volgograd who died in 1999 [20]), the Japanese encephalitis virus Beijing-1 strain (both obtained from the State Virus Collection of the Ivanovsky Institute of Virology, Russian Academy of Medical Sciences), and strain 205 of the tick-borne encephalitis virus (TBEV) from the Collection of State Research Center of Virology and Biotechnology "Vector". NS0 murine myeloma, Vero, and pig embryo kidney cell cultures (cell culture bank of "Vector") were grown in DMEM and DMEM/F12 (Gibco, USA) supplemented with 10% calf fetal serum (Gibco) and 80 μg/ml gentamicin sulfate. Viruses were purified by centrifugation in a sucrose density gradient [21]. All experiments with pathogenic viral material were carried out in BSL-3 conditions providing maximal biological defense of personnel and the environment.

Preparation of recombinant fragments $E_{260\text{-}466}$: cloning, expression, and purification. RT-PCR (primers 5′AAGCAGTCTGTATAGCATTGGG3′-WNF and 5′CCCCAGCAATCCCTGCGTTATCC3′-WNR) was used to amplify the cDNA fragment 1712-2333 bp of WNV (LEIV-Vlg99-27889-human, Gene bank locus, AY277252). The cDNA fragment was purified using the S.N.A.P. gel purification kit (Invitrogen, USA) and cloned in pCR/T7/NT (Invitrogen) to yield recombinant plasmids pCR-2E[dIII]. These plasmids contain the cDNA fragment coding for the WNV $E_{260\text{-}466}$ fragment with molecular weight of 22.7 kDa. *Escherichia coli* cells BL21(DE3)pLysS (Invitrogen) were transformed with these pCR-2E[dIII] plasmids. The recombinant protein synthesis was induced by adding isopropyl-D-thiogalactoside to the cell cultures at A_{600} = 0.5-0.8 to the final concentration of 2 mM, followed by incubation at 37°C for 4-6 h. The recombinant proteins containing a polyHis block were purified by affinity chromatography on Ni-

NTA agarose (Qiagen, Germany) as recommended by the manufacturer. The quality of the recombinant peptides was confirmed by electrophoresis in 15% polyacrylamide gel [22], and the concentration of peptides was determined by the Bio-Rad Protein Assay (Bio-Rad, USA).

Polyclonal and monoclonal antibodies. Mouse polyclonal antibodies against WNV were obtained as described previously [23]. To obtain the mouse polyclonal antibodies against the recombinant polypeptide $E_{260-466}$, BALB/c mice were immunized using a similar scheme. NS0 mouse myeloma cells were hybridized with the splenocytes of immunized mice. Thus we obtained 200 hybrid cell lines growing on a selective medium with aminopterin. Using EIA, we selected seven hybridomas producing MAbs against the recombinant polypeptide $E_{260-466}$. Hybridomas, which displayed stable MAbs secretion, were cloned twice and frozen. The Ig classes were identified by EIA with monospecific sera (Sigma, USA).

EIA and immunoblotting. EIA was carried out as described previously [24]. To study the interactions with MAbs, 150 ng of $E_{260-466}$ or 200 ng of purified WNV, JEV, or TBEV were added to each well of a polystyrene EIA plate. In EIA, A_{492} was measured using a Uniscan spectrophotometer (Finland). Immunoblotting was performed as in [25, 26]. The protein concentration during the preparation of the recombinant polypeptides and purified WNV was measured using the protein assay kit (Bio-Rad). Ig-biotinylation was carried out as in [27].

Competitive EIA. $E_{260-466}$ was sorbed on polystyrene plates (150 ng/well) overnight at 20°C. Nonspecific binding was blocked by 0.5% casein solution. Subsequent incubation with MAbs (1 : 100-1 : 200) was performed overnight at 20°C. Then the biotinylated MAbs were incubated for 1 h at 37°C. Immune complexes were detected using streptavidin–peroxidase. The level of competition was evaluated as follows: total competition (reduction of $E_{260-466}$–biotinylated MAbs binding more than 75%), partial competition with maximal effect (75-50%), partial competition with minimal effect (50-25%), and absence of competition.

Neutralization reaction and hemagglutination inhibition assay. Neutralization reaction of WNV was performed by a micromethod using Vero cells [28]. As controls, we used normal serum and hyperimmune serum against WNV. The results were recorded after 4 days. Each dilution was tested with at least four wells with cell monolayers. Hemagglutination inhibition assays were carried out in round-bottom 96-well plates as in [29] using WNV at eight hemagglutinating units.

Modeling of WNV gpE 3-D structure. Protein Data Bank results of the WNV gpE domain III spatial structure obtained by NMR were used for modeling of the 3D WNV gpE spatial structure [13]. 3D prediction program ESyPred3D [30] was used for the reconstruction of the WNV gpE atomic coordinates. Data concerning the TBEV (PDB ID 1SVB) and dengue virus (PDB ID 1OKE) protein E 3D structures were applied as the reference samples. 3D structure superposition was performed using the MultiProt program [31]. Spatial structures similarity was evaluated using similarity measure such as root mean square deviation (RMSD).

Sample preparation for force interaction measurements. The procedure of tip and sample functionalization has been described in detail [32, 33]. Covalent attachment of proteins onto the tip and sample surfaces without special linkers was used. Silicon nitride tips were functionalized for 15 min in 1% solution of glutaraldehyde (Sigma). After rinsing, the tips were immersed into a solution of integrin $\alpha V\beta 3$. The protein-functionalized tips were used immediately for measurements. Substrates (freshly cleaved muscovite mica) were functionalized for 7 min in a 1% v/v 3-aminopropyltriethoxysilane (APTES) (Sigma) solution in water and for 15 min in a 1% v/v glutaraldehyde solution in water. After rinsing with deionized ultra high quality water, the samples were immersed into a solution of the $E_{260-466}$ fragment. The concentration of proteins was 250 mg/liter in both cases. The times of immobilization were 15 min. The nonreacted and loosely bound proteins were subsequently removed by extensive washing with phosphate-buffered saline (PBS) (50 mM phosphate buffer with 150 mM NaCl, pH 7.4, 25°C).

Atomic force spectroscopy. Atomic force microscope Nanoscope IV Picoforce (Veeco Instruments, USA) in force–volume mode was used. Sharpened V-shaped silicon nitride probes (NP-S) were used. All experiments were performed in PBS using instrumental loading rates from 300 to 1000 nm/sec (force loading rates were 18-60 nN/sec for a nominal cantilever spring constant of 0.06). The spring constant of each cantilever was calibrated using the built-in calibration procedure of the Nanoscope IV Picoforce AFM based on the thermal fluctuations method [34]. Fuzzy logic-based software [35] to process the experimental data semiautomatically was used for obtaining sets of "specific interaction" events including the force and loading rate values and force histograms. The dependences of the specific interaction force on the force loading rate were obtained using OriginPro software.

RESULTS

3D models of flaviviral gpE C-end and production of WNV recombinant polypeptide $E_{260-466}$. The 3D model of the WNV gpE was proposed from the theoretical modeling (Fig. 1a). RMSD data on pairwise comparison of protein E full-size structures had the following values: for dengue virus and TBEV, 2.2 Å; for dengue virus and WNV, 2.7 Å; for TBEV and WNV, 1.9 Å. On comparison of the protein E C-end fragment spatial structures RMSD data were 2.5, 2.7, and 1.9 Å, respectively. The data are

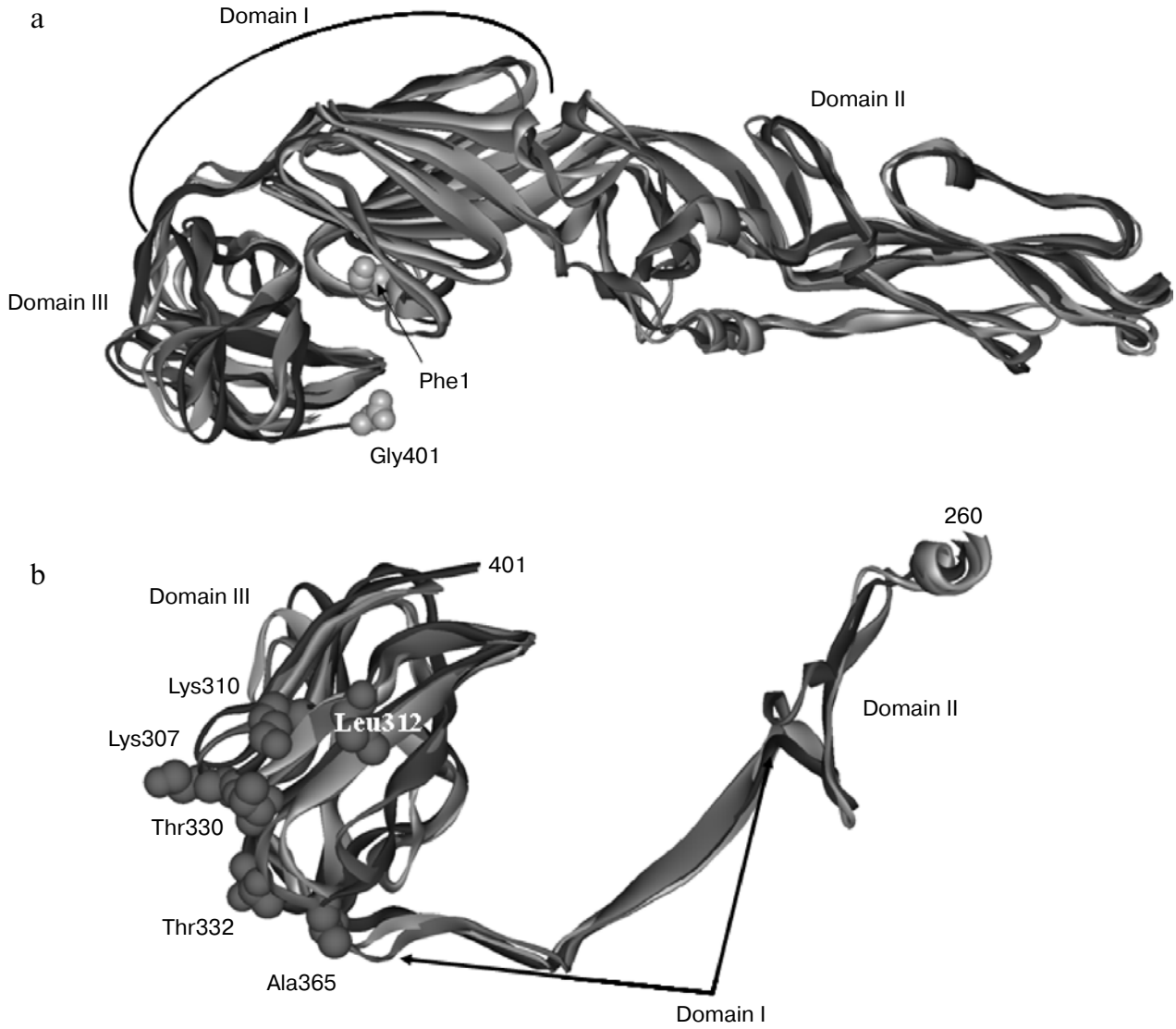

Fig. 1. Alignment of tertiary structures: a) glycoproteins E of TBEV (PDB Id 1svb), dengue-2 virus (PDB Id 1oke), and WNV (PDB Id 1s6n); b) C-end fragments of glycoproteins E of TBEV (PDB Id 1svb), dengue-2 virus (PDB Id 1oke), and WNV (PDB Id 1s6n). Glycoproteins E are shown in light gray (TBEV), gray (dengue-2), and dark gray (WNV).

evidence of high degree of structural similarity between full-size proteins E and C-end protein E fragments of these proteins. Spatial similarity of these proteins suggests that the biological functions of protein E are inherent in the same regions of this protein. This enables us to use the previously obtained data concerning the functions of different flaviviral gpE regions, especially domain III, for the choice of such WNV gpE fragment that should be immunologically and functionally significant. The selected polypeptide contains 260-466 a.a. of protein E ($E_{260-466}$) thus including: (i) the fragment of domain II causing the production of protective antibodies, (ii) the whole domain III containing the classic receptor region, and (iii) the well known conformational epitopes inducing viral neutralizing antibodies [11-16]. The modeling of this fragment showed (Fig. 1b) that the conformation of the described gpE C-end fragment is similar to the corresponding region of the full-size gpE.

The recombinant polypeptide $E_{260-466}$ was obtained using the gene coding gpE of the modern WNV Russian strain LEIV-Vlg99-27889-human by the methodology described above. The high degree of $E_{260-466}$ affinity purification was confirmed by electrophoresis (Fig. 2). The molecular weight of $E_{260-466}$ predicted by the computer code Vector NTI [36] is about 22.7 kDa, which is similar to the electrophoresis data.

Testing of $E_{260-466}$ antigenic structure. Interaction of the recombinant polypeptide $E_{260-466}$ with the polyclonal murine antibodies against the purified WNV and the rabbit antibodies against the purified TBEV was detected by EIA

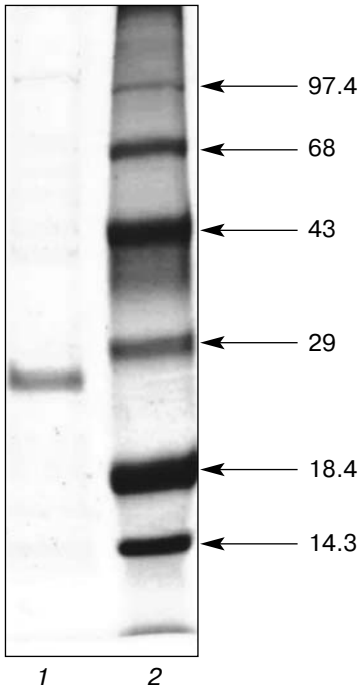

Fig. 2. Electrophoretic patterns of recombinant polypeptide $E_{260-466}$. Lanes: *1*) recombinant polypeptide $E_{260-466}$; *2*) molecular weight markers, kDa.

(Fig. 3a). Polyclonal murine antibodies against $E_{260-466}$ recognize WNV gpE in immunoblot (Fig. 3b), which indicates the immunologic similarity of the antigen structure between the WNV native gpE region of 260-466 a.a. and the produced recombinant $E_{260-466}$. The ability of the polyclonal antibodies against TBEV to recognize the WNV recombinant polypeptide $E_{260-466}$ epitopes is evidence for common gpE C-end epitopes of TBEV and WNV.

Investigation of immunochemical properties of MAbs against $E_{260-466}$. Data characterizing the immunochemical properties of MAbs against $E_{260-466}$ are presented in the table. It is seen that the MAbs interact with the $E_{260-466}$ and WNV native gpE in EIA and immunoblot quite effectively. Only MAb 3A6 fails to react with the purified WN virions in immunoblot. The similarity of the recombinant polypeptide $E_{260-466}$ and corresponding region of the native gpE is proven by these results. It was shown by the neutralizing activity of MAbs 3B9 and 4C10 that the recombinant polypeptide retained the epitopes inducing virus-neutralizing antibodies. In addition, MAbs 3B9 are able to inhibit the hemagglutination (table), which is evidence for an important role of the epitope recognized by these antibodies in the virus–host cell interaction.

To summarize, the study of immunochemical properties using polyclonal as well as seven types of MAbs against $E_{260-466}$ confirms the immunochemical similarity of the recombinant polypeptide $E_{260-466}$ and the native WNV gpE 260-466 a.a. region. It also supports the conclusion that the proposed spatial 3D model of $E_{260-466}$ correctly describes the spatial organization of the gpE C-end of the recombinant $E_{260-466}$.

Mapping of epitopes recognized by MAbs. Cross reactivity of MAbs. A wide variety of the MAbs immunochemical properties lead us to propose that all obtained

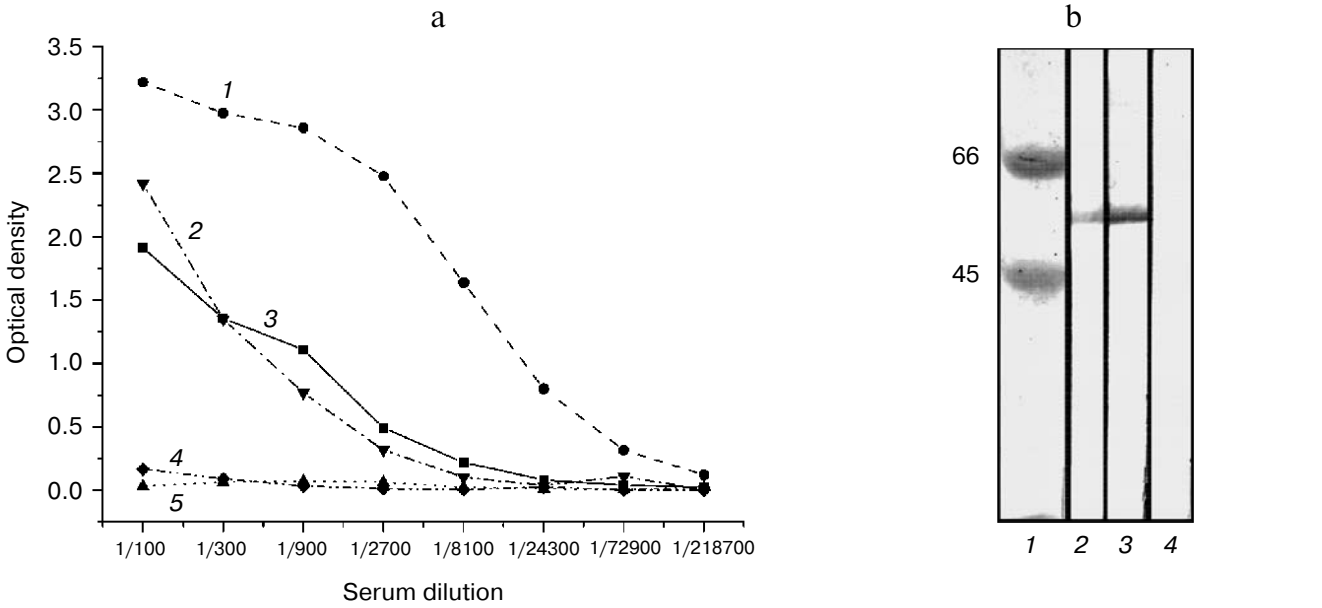

Fig. 3. a) Study of interaction of recombinant polypeptide $E_{260-466}$ with various polyclonal antibodies (PAbs) by EIA: *1*) murine anti-$E_{260-466}$ PAbs; 2) rabbit anti-TBEV PAbs; *3*) murine anti-WNV PAbs; *4*) control experiment with normal rabbit serum; *5*) control experiment with normal murine serum. b) Immunoblot of WNV gpE with murine polyclonal antibodies: *1*) molecular weight markers, kDa; *2*) murine anti-WNV PAbs in dilution 1 : 500; *3*) murine anti-$E_{260-466}$ PAbs in dilution 1 : 500; *4*) control experiment with normal murine serum.

Monoclonal antibodies against recombinant fragment $E_{260-466}$

MAbs	MAbs titer in EIA				Western blot data*		Ig class	MAbs titer in NR	MAbs titer in HAI
	$E_{260-466}$	WNV	TBEV	JEV	$E_{260-466}$	WNV			
5A4	900	72 900	–	–	+	+	IgG1	–	–
3B9	218 700	218 700	–	–	+	+	IgG 2b	320	80
4C10	218 700	218 700	72 900	–	+	+	IgM	640	–
8D9	218 700	218 700	72 900	–	+	+	IgM	–	–
8G8	24 300	72 900	–	8100	+	+	IgG3	–	–
3A6	218 700	218 700	–	900	+	–	IgG1	–	–
6H4	1 800 000	218 700	–	2700	+	+	IgG1	–	–

Note: NR, neutralization reaction; HAI, hemagglutination inhibition assay.
* Sign (+) indicates interaction between antigen and antibodies revealed by Western blot.

MAbs recognize certain unique antigenic determinants both in the recombinant polypeptide and in the full-size viral gpE of WNV. Competitive EIA showed that there is no competition between the antibodies except for MAbs 5A4 and 3B9. A typical picture of the competitive EIA results is demonstrated in Fig. 4 with the example of MAbs 5A4 labeled by biotin.

The cross reactivity and competitive EIA data showed that the MAbs can be divided into three groups. MAbs 8D9 and 4C10 provide cross reactivity with TBEV (table) and are included in group I. These two MAbs do not compete with each other and therefore they recognize different epitopes of gpE. The antigen determinant recognized by MAb 4C10 can induce the synthesis of virus-neutralizing antibodies. MAbs 8G8, 3A6, and 6H4, which are cross-reactive with JEV, belong to group II (table), and they were not able to compete with each other, thus they recognize different epitopes. Group III consists of MAbs 5A4 and 3B9 interacting with species-specific epitopes, in addition MAb 3B9 that has virus-neutralizing activity. Partial competition with the maximal effect between these antibodies was shown by competitive EIA. The effect of the labeled MAb 5A4 binding with $E_{260-466}$ was lowered by 58% due to the competition with MAb 3B9 (Fig. 4). Thus epitopes distinguished by MAbs 5A4 and 3B9 are partially overlapping and located rather close to each other. To summarize, these results allow us to identify no less than seven various epitopes in the structure of the $E_{260-466}$ region of WNV gpE.

Atomic force spectroscopy. Figure 5a shows a non-normalized histogram demonstrating the experimental distribution of the specific interaction forces between αVβ3 integrin and recombinant peptide $E_{260-466}$ corresponding to the loading rate in the range 2-20 nN/sec. It has a well-defined peak at about 80 ± 15 pN, and a small second peak can be discerned at 140 ± 28 pN that can be possible at double bond rupture. These frequencies of events were significantly different. Frequency of appearance of the 80-pN peak was 15.1%, while the frequency of the 140-pN peak was only 0.8% from the total number of measured force curves. Figure 5b shows the results of control experiments.

We measured the adhesion force between peptide $E_{260-466}$ and cell receptor LBP interacting with domain II of WNV [8]. The histogram shows the absence of specific interaction for this case. Similar data was obtained for other control experiments including, e.g. the partial systems of the type "functionalized AFM tip–unfunctionalized sample", etc. Figure 6 shows the dependence of the specific interaction between $E_{260-466}$ and integrin αVβ3 on the force loading range. Based on the Bell–Evans model

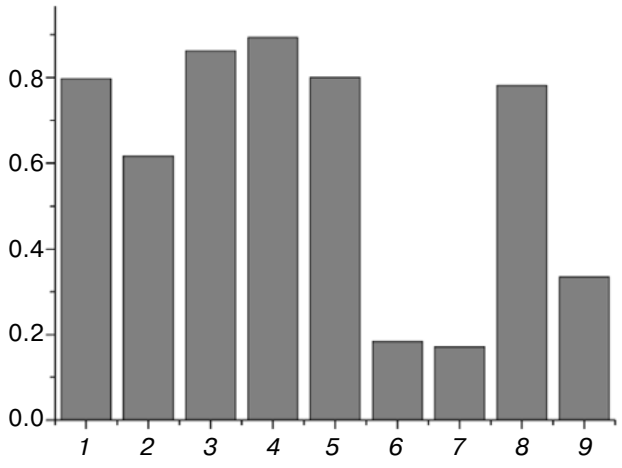

Fig. 4. Mapping of WNV $E_{260-466}$ region epitopes. Competition between MAbs 5A4 labeled by biotin and 3B9. The abscissa shows MAbs used for preliminary incubation with $E_{260-466}$. Columns: *1*) MAbs 3A6; *2*) MAbs 6H4; *3*) MAbs 8G8; *4*) MAbs 8D9; *5*) control experiment without preliminary incubation; *6*) murine anti-WNV PAbs; *7*) MAbs 5A4; *8*) MAbs 4C10; *9*) MAbs 3B9. Antibodies dilution was 1 : 100, 2 μg/well. MAbs 5A4 labeled by biotin dilution was 1 : 200, 0.2 μg/well.

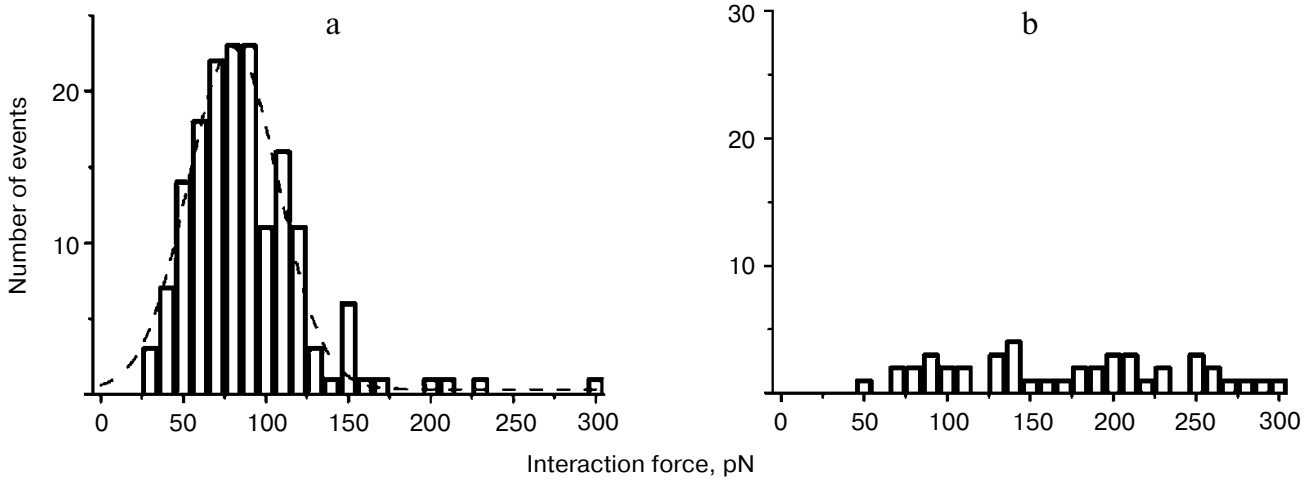

Fig. 5. a) Histogram demonstrating experimental distribution of specific interaction forces for $E_{260-466}$–αVβ3 integrin pair. b) Histogram of control experiment demonstrating interaction between LBP and $E_{260-466}$.

[36], the experimental data are well approximated with a single straight line whose processing gives the value of the dissociation barrier thickness of 0.32 nm and the value of 0.76 sec^{-1} for the dissociation rate at zero force. Both values are typical for the averaged parameters of ligand–receptor binding of protein molecule measured by AFM [37].

DISCUSSION

Glycoprotein E presented on the flaviviral particle surface as a homodimer has a conservative spatial structure [6]. This protein and its recombinant analog can induce formation of full antiviral immunity [38]. It was shown by experiments with shorter protein E fragments that WNV protein E domain III fragment 303-395 a.a. and/or dengue virus protein $E_{296-400}$ are important for immunity formation in principle [11, 39]. Our data confirm these previously published data indirectly. Moreover, the detected immunochemical similarity between the $E_{260-466}$ and gpE unambiguously attests that the $E_{260-466}$ protein is a very prospective candidate for elaboration of a vaccine against WNF.

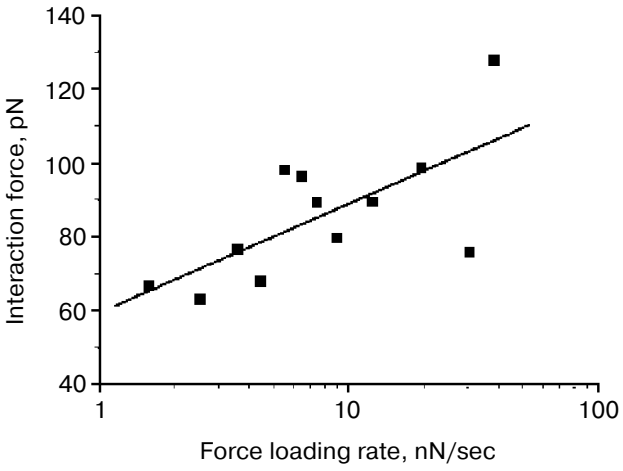

Fig. 6. Dependence of specific interaction between $E_{260-466}$ and αVβ3 integrin on force loading rate.

The positions of different $E_{260-466}$ epitopes were mapped by the construction of recombinant polypeptides modeling this region and investigation of their interactions with MAbs. Earlier this approach was successfully used for the mapping of the WNV gpE 1-180 region containing the fusion peptide [8]. A similar strategy was applied in the present work: the $E_{260-466}$ protein that includes the receptor epitope interacting with cell membrane αvβ3-integrin and epitopes inducing virus-neutralizing antibodies was created.

Data on virus-neutralizing activity of MAbs 3B9 and 4C10 created against polypeptide $E_{260-466}$ containing domain III confirm earlier published results about induction by this region of protein E virus-neutralizing antibodies [14, 40, 41]. The computer algorithm for prediction the location of immunologically significant epitopes of WNV protein E showed the presence partially overlapping epitopes for virus-neutralizing antibodies E16 and E24 within domain III of WNV [42, 43]. However, we did not detect a significant competition between MAbs 3B9 and 4C10, which suggests that these MAbs recognize epitopes that are distinct from epitopes of MAbs E16 and E24.

Competitive EIA results show that only two types of MAbs (5A4 and 3B9) compete between themselves for epitope bindings of antibodies. The other MAbs did not compete among themselves. Earlier described MAbs usually competed among themselves for the domain III epi-

topes, which suggested partial overlap of epitopes for these MAbs types and limitation of the area of location of these epitopes [42, 43]. Detected MAb cross reactivity concerning TBEV and JEV and the presence of antihemagglutinin activity of MAbs is in good agreement with data published earlier [44].

In our work, MAbs have been obtained against recombinant fragment $E_{260\text{-}466}$ of WNV protein E and, most likely, it has predetermined wider immune recognition of epitopes of this polypeptide. The possible practical significance of these data making the use of recombinant polypeptide as an antigen for diagnostic test systems and the prepared MAbs panel for creation of diagnostic sets is clear.

A functional feature of WNV domain III is the presence of a receptor region whose blocking can prevent the penetration of the virus into a host cell. It was shown by the neutralization reaction that two types of the constructed MAbs (3B9 and 4C10) do inhibit the viral infection of Vero cells. Subsequent research on the viral neutralizing mechanism of given antibodies can bring new arguments for the confirmation of the interaction of the MAbs with the canonic WNV domain III RGD motif. Recently it was demonstrated that the RGD motif does not mediate the WNV–WNV receptor (αVβ3-integrin) interaction [17]. Thus an alternative theory of inhibition of the penetration of WNV into a host cell, which takes into account the binding of MAbs with an unidentified epitope thus providing an interaction with αVβ3 integrin, is to be considered. The antigenic similarity of the recombinant gpE C-end fragment with the corresponding native region suggests the possibility of using $E_{260\text{-}466}$ for studies on virus–host cell interaction.

Recently, a complex of immunochemical and AFM methods was used to study the LBP–WNV gpE domain II interaction [12]. The interaction specificity was proven and the specific single molecule force was measured as 105 ± 20 pN. Besides, the WNV recombinant $E_{260\text{-}466}$ protein interacts with αVβ3 integrin, and the corresponding specific interaction force was measured previously as 45 ± 5 pN [17, 18]. Also significantly rarer interactions of force 83 pN (presumably 2×45) and 124 pN (presumably 3×45) have been registered. The authors assumed that their occurrence is caused by interactions between two or three pairs of molecules. The recombinant WNV protein E domain III included 303-395 a.a. of WNV protein E, and its molecular weight was approximately 12 kDa. In our experiments, we used recombinant polypeptide of WNV protein E 260-466 a.a. with molecular weight near 23 kDa. The force of interaction for this protein with $\alpha_V\beta_3$ integrin was 80 ± 15 pN. Interactions with force 140 ± 28 pN were observed approximately 19 times less than proposed double interactions with force 2×80 pN. Proposed threefold interactions with force near 200-240 pN have not been registered. Frequency of occurrence of double events was eight events during 1000 measurements. This suggests that for authentic registration of a threefold event it would be necessary to increase the number of measurements by approximately 100-fold. Another possible explanation is that in our experiments we used polypeptide having molecular weight almost twice larger than was used earlier. Probably the large sizes of molecules could significantly decrease the frequency of occurrence of double and triple interactions for steric reasons. Also studies of the immunochemical properties of this polypeptide have shown its epitope identity to corresponding regions of the full-size molecule of protein E. These experimental data have been confirmed by theoretical analysis (Fig. 1b). It also has confirmed the existence of the selected fragment of protein E in a conformation that is as much as possible similar to the appropriate region of the full-size protein E. Probably these structural features have predetermined more effective interaction of these molecules.

Unfortunately, the question of the possibility of estimating the interaction force of the WNV protein domain III with αVβ3 integrin for conditions when protein E is a part of a virus particle still remains open. Technical complexities of operation with a virus particle, its size, complexity of structure, and presence lipid membranes make these experiments, and especially the analysis of their results, a separate scientific task. It is also impossible to eliminate purely technical reasons for observable distinctions of values of specific interaction force. Quite possibly, they can be linked to small differences in the conditions of carrying out AFM experiments. However the fact of the presence of ligand–receptor interactions and the characteristic of the interaction forces are especially important for development of further studies of receptor interaction mechanisms between a virus and a cell.

Thus, our data suggest a more complex model of WNV penetration into the host cell. We propose that WNV gpE can specifically interact with two cellular proteins (LBP and αVβ3 integrin) during virus entry. Most likely, WNV protein E domain III interacts with αVβ3 integrin at an initial stage of entry of WNV into a cell. During pH-dependent endocytosis there is a joint interaction of the WNV protein E domain II, namely protein E fusion peptide (cd-loop) to a cellular membrane and protein E bc-loop with co-receptor LBP. The complicated mechanism of interaction of a virus and a cell suggests that detailed study of this process is rather promising for the future.

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