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Viewpoint

Computer Analysis of Antigenic Domains and RGD-like Sequences (RGWG) in the E Glycoprotein of Flaviviruses: An Approach to Vaccine Development

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Abstract

Antigenic domains and RGD-like sequences in the E glycoprotein of the flaviviruses Japanese encephalitis virus, yellow fever virus, West Nile virus, dengue type 4 virus, and tick-borne encephalitis virus were analyzed by computer programs that provide information on the physical properties of the polypeptides. The use of computer programs for the development of vaccines based on the synthesis of antigenic peptides is discussed. Synthetic viral peptides are proposed to be used for topical application so as to interfere with the virus-cell interaction. Viral peptides with antigenic epitopes to protect against dengue virus infection without enhancing pathogenesis may also be developed on the basis of the computer analysis.

Introduction

Dengue viruses have the widest geographical distribution and the highest morbidity among more than 70 members of the flavivirus family (Flaviviridae). In addition to the classical dengue syndrome, in dengue epidemic areas severe forms of

the disease with hemorrhagic fever shock syndrome occur in infants and young adults (1).

Dengue virions contain the capsid protein (designated C, 13–15 kD in size), the membrane-like protein (M of 8 kD), and the envelope protein (E glycoprotein of 51–59 kD). The E glycoprotein is present on the surface of the virion and is responsible for virion adsorption to host cells, hemagglutination, and reactivity with neutralizing antibodies. Dengue type specificity was also assigned to the E glycoprotein (summarized in 1).

In the absence of licensed vaccines for the prevention of dengue, the World Health Organization appointed a Steering Committee on Dengue to establish a program for dengue vaccine development (2). The report of the scientific meeting on current approaches to the development of dengue vaccines was read during the VII International Congress of Virology in 1987 and was published in 1988 (2). It was distributed in 1989 at the 2nd International Symposium on Positive-Strand RNA viruses organized by Drs. C. Kunz and F. X. Heinz in Vienna. The two WHO-sponsored meetings (1987 and 1989) were devoted to the epidemiology of flavivirus-caused diseases in the world, and the ongoing virological and molecular studies that could lead to the development of safe and potent dengue vaccines.

The Strategic Plan for Dengue Vaccine of the WHO program for vaccine development (2) encompasses all possible current approaches to vaccine development, from selection of attenuated viruses to the molecular definition of virus genes, and from the analysis of antigenic epitopes in the E glycoprotein to the development of recombinant vaccinia/dengue virus, as well as proteins synthesized by baculovirus and synthetic antigenic peptides.

The present study deals with my idea that computer analysis of the properties of viral glycoproteins will make it possible to design synthetic viral antigenic peptides that can be applied directly to the skin. Such peptides may interfere with virus infection either by inhibiting virus adsorption to cells in the skin or by stimulating local and general antiviral immunity in the skin and the draining lymph nodes.

The usefulness of recombinant vaccinia virus as a carrier of dengue virus E glycoprotein gene was summarized in the report of the 1987 WHO meeting (2). It was indicated that infection of mice or hamsters with recombinant vaccinia/dengue virus did not result in the production of detectable anti-dengue antibodies. When these hamsters were later injected with live dengue type 2 virus, a secondary antibody response to dengue virus occurred, indicating that antibodies had been synthesized as a result of infection with a recombinant vaccinia virus. However, preliminary studies of the recombinant vaccinia/dengue virus in monkeys did not reveal good production of anti-dengue antibodies or a significant reduction in the effects of the antibodies on a challenge dose of 10^5 cell culture infectious doses of dengue virus. It was also indicated that "a recombinant vaccinia virus expressed Pr M, E and NS1 in infected cell cultures . . . but antibody responses, however, were not encouraging in cotton rats infected with the recombinant vaccinia virus" (2). The dilemma posed by these unsuccessful attempts to vaccinate

test animals with recombinant vaccinia/dengue virus might be related to the ability of the recombinant vaccinia virus to interact with the skin Langerhans cells at the site of virus inoculation. It was reported by Nagao and Inaba (3) that vaccinia virions are taken up by Langerhans cells, which process the viral antigens, and then travel to the lymph nodes, where they present the viral antigens to T lymphocytes. It would appear that the dengue virus glycoproteins produced by the vaccinia virus recombinants do not reach the Langerhans cells, since they remain in the infected keratinocytes in the skin. The involvement of Langerhans cells in virus infections in the skin was recently summarized and reviewed (4).

The involvement of skin cellular elements, especially Langerhans cells, in natural infection by flaviviruses has not yet been studied, even though it is known that "Initial virus replication takes place in the tissue adjacent to the (insect) bite site as well as in regional lymph nodes. Virus is transported by the lymphatic system to the thoracic duct and then enters the blood" (from ref. 5 quoting D. Malkova, ref. 6). It was reported by T.P. Monath (7) that "In Macaca mulatta monkey inoculated subcutaneously, dengue virus was found before detectable viremia in the skin (8), at the inoculation site and in both regional and remote lymph nodes indicating rapid dissemination. Two or three days after onset of viremia, virus appeared in multiple skin sites and many other tissues."

Recent studies on skin Langerhans cells and the dendritic cell system revealed that progenitor cells in the bone marrow produce dendritic cells that migrate via the blood into epithelial layers in all organs of the body. Here the dendritic cells terminally differentiate and function (reviewed in 4). Skin Langerhans cells were found to interact with invading viruses and serve as a key mechanism in the skin defense against virus infection (reviewed in 4). Thus, protection against dengue virus infection in the skin at the site of an insect bite requires understanding of the role of Langerhans cells and Thy-1-positive skin dendritic cells in the skin immune response to flaviviruses.

Langerhans cells in the skin are able to interact not only with viruses, but also with soluble substances, including antigens present in the saliva released into the skin during a mosquito bite (9). These cells were found to interact with herpes simplex virus-1 (HSV-1) that is carried to the lymph nodes (10). Since Langerhans cells interact with soluble antigens, it might be possible to assume that they will be able to interact with synthetic viral antigenic peptides modeled according to the epitopes in dengue virus glycoprotein E that interact with cell receptors and neutralizing antibodies. The properties of such synthetic peptides are the subject of the present study.

Recently, Roehrig et al. (11) utilized a computer program for the prediction of antigenic domains in Murray Valley fever virus glycoprotein E and synthesized peptides that were able to stimulate the production of antiviral antibodies when injected into rodents. Mandl et al. (12) presented a model of the tick-borne encephalitis virus envelope glycoprotein E and correlated the antigenic domains to defined amino acid sequences. Mason et al. (13) studied the antigenic and neutralization domains in the amino acid sequence 280–414 in the E glycoprotein of

Japanese encephalitis virus (JEV). This peptide contains the RGD sequence. Antisera raised against a fusion protein containing aa 280–414 of the JEV E glycoprotein did not exhibit any plaque reduction or neutralization activity.

Computer analysis of flavivirus glycoprotein E revealed a conserved amino acid sequence RGWG in all viruses. In addition, Japanese encephalitis virus and yellow fever virus also have the RGD sequence that is the cell-binding domain in fibronectin. Recent studies showed that the RGD synthetic peptide interferes with the binding of foot-and-mouth disease virus (FMDV) to cellular receptors (14, 15). The presence of RGW and RGD sequences in flavivirus glycoprotein E seems to suggest that these amino acid sequences are involved in virus adsorption to cells and that suitable synthetic peptides will interfere with flavivirus skin infection at the site of the insect bite.

The flavivirus family includes viruses that cause yellow fever, Japanese encephalitis, tick-borne encephalitis, West Nile fever, and dengue fever. The availability of the amino acid sequences of the E glycoproteins in computer programs allows the prediction of antigenic domains in the viral proteins and the modeling of common and unique putative antigenic domains. Immunization with synthetic viral peptides applied to the skin and taken up by Langerhans cells may succeed in protecting against flavivirus infection in endemic areas around the world. Introduction into the skin of a synthetic peptide (like RGD or RGW) that may be able to interfere with adsorption of virions to cellular receptors might be another approach to protect humans against flavivirus-caused diseases.

Methods

Primary amino acid sequence

The primary amino acid sequences of the E glycoproteins studied were obtained for Japanese encephalitis virus (JEV) (16), West Nile virus (WNV) (17), yellow fever virus (YFV) (18), dengue virus (DV) (1), and tick-borne encephalitis virus (TBEV) (19). Additional amino acid sequences for each virus were obtained through the NBRF Protein Sequence Data Bank from the NBRF Protein Identification Resource in the University of Wisconsin Genetics Computer Group (GCG) software (20).

Computer programs

The compilation of seven algorithms in one program by Wolf et al. (21) provides information for analyzing the properties of amino acids (aa) in a polypeptide chain by using the primary aa sequence of the peptide. The following properties of aa were studied: a) hydrophilicity according to Hopp and Woods (22, 23) or Kyte and Doolittle (24), b) surface probability according to Emini et al. (25), c) chain flexibility according to Karplus and Schulz (26), d) secondary structure according to Chou and Fasman (27) and Garnier-Osguthorpe-Robson (28), and e) the antigenic-

ity index according to Wolf et al. (21). All of these provide quantitative estimates for hydrophilicity, surface probability, chain flexibility, and the antigenicity index. The program compiled by Wolf et al. (21) is available in the software of the University of Wisconsin GCG.

The predicted antigenic domains were determined on the basis of the highest value of surface probability and a high hydrophilicity value, provided the sequence had β-turn or α-helix conformations, as predicted by both the Chou-Fasman and Garnier-Osguthorpe-Robson methods. A high antigenicity index value provided an indication for the location of the antigenic domain. The predicted secondary structure was also taken into account, since β-turn domains in polypeptides were found to have a better antigenicity value than β sheets. To obtain a numerical value for the property of each amino acid in a putative antigenic domain, the normalized value was determined by summing up the values of all amino acids and dividing them by the number of amino acids in the domain. This normalized value per amino acid was used for the comparison of different putative antigenic domains.

Results

Putative antigenic domains in the E glycoprotein of JEV, WNV, YFV, and DV

With the help of the computer program developed by Wolf et al. (21), each of the E glycoproteins of the above viruses were analyzed and the putative antigenic domains in the primary amino acid sequence was determined. An antigenic domain is defined by a high averaged hydrophilicity value, a high surface probability and flexibility values, a β-turn conformation or an α-helix conformation, and a relatively high antigenicity index. If all these properties are found in an antigenic domain, it is considered the most probable and the others are arranged in a decreasing order. Such an analysis of the JEV E glycoprotein is presented in Table 1. Fourteen putative antigenic domains were found, one in the cytoplasmic portion of the JEV E glycoprotein and the rest in the external part of the protein. It is indicated that putative antigenic domain #4 contains the conserved sequence RGWG and putative antigenic domain #13 contains the sequence GRGD, which in fibronectin is the amino acid sequence that binds to integrin, the cell surface protein receptor of fibronectin (29). The five most probable antigenic domains are #2, #3, #5, #9, and #10 based on the highest normalized surface probability values.

Table 2 provides the analysis of WNV E(V3) glycoprotein in which 11 putative antigenic domains were identified by computer analysis. It was also noted that in putative antigenic domain #4, the amino acid sequence RGWG is present. The most probable antigenic domains are #3, #6, #2, and #8, as determined by the surface probability value.

Table 3 shows the 15 putative antigenic domains in the E glycoprotein of YFV. Putative antigenic domain #5 contains the amino acids RGWG, and putative antigenic domain #13 contains the amino acid sequence GRGD.

Table 1. Putative antigenic domains in the envelope protein E of Japanese encephalitis virus (16)

Antigenic domain	No. of aa (aa position)	Hydrophilicity per aa	Surface probability per aa	Flexibility per aa	Conformation		Antigenic index per aa
					CF	GOR	
1	7 (6–12)	0.74	2.4	1.01	t+T	T+B	0.96
2	8 (35–42)	0.88	9.95 (1)	0.90	t+H	—	0.87
3	14 (75–88)	1.73	9.7 (2)	1.05	H	H	1.05
4	9 (95–103)[a]	1.34	2.13	1.03	B+T	B+T	1.25
5	9 (129–137)	1.13	6.86 (3)	1.02	T	T	1.07
6	7 (180–186)	2.54	1.72	1.01	T	T	1.01
7	8 (190–197)	1.12	2.96	1.03	T	T	1.17
8	8 (212–219)	0.74	3.05	0.96	H	H	0.54
9	14 (225–238)	1.09	5.63 (4)	0.96	T+t	T+H	1.03
10	11 (284–295)	0.49	5.62 (5)	0.99	b+H	H	0.63
11	15 (303–317)	0.71	2.86	1.01	t+T	T	0.81
12	7 (374–380)	0.58	2.13	1.01	H+T	B+T	1.16
13	17 (387–403)[b]	1.39	4.81	1.01	h	T	0.92
14	7 (473–479)[c]	0.93	5.65	1.12	t	H	0.89

[a]RGWG.
[b]GRGD.
[c]Intracytoplasmic.
CF = Chou-Fasman (27); GOR = Garnier-Osguthorpe-Robson (28); t,T = β-turn; b,B = β-sheet; h,H = alpha helix.

Table 2. Putative antigenic domains in membrane protein V3 of West Nile virus (18)

Antigenic domain	No. of aa (aa position)	Hydrophilicity per aa	Surface probability per aa	Flexibility per aa	Conformation		Antigenic index per aa
					CF	GOR	
1	8 (5–12)	0.68	3.13	1.01	T	T	1.02
2	7 (35–41)	1.04	11.10 (3)	1.03	T+h	T+h	1.11
3	10 (78–87)	1.42	12.69 (1)	1.03	H	H	0.87
4	9 (95–103)[a]	0.76	1.29	0.92	T	T	0.98
5	9 (129–137)	0.94	9.50 (4)	1.00	H	B	0.70
6	8 (227–234)	1.87	12.49 (2)	1.04	b+H	—	0.90
7	9 (237–245)	1.31	5.17	1.01	H	H	0.93
8	12 (286–297)	0.67	7.15 (5)	1.01	H	H	0.89
9	13 (385–397)	1.86	4.88	1.01	t	T	1.20
10	6 (407–412)	0.55	2.43	1.02	b+H	B	0.80
11	8 (470–477)[b]	0.85	5.12	0.99	b+t	T	0.92

[a]RGWG.
[c]Intracytoplasmic.
CF = Chou-Fasman (27); GOR = Garnier-Osguthorpe-Robson (28); t,T =. β-turn; b,B = β-sheet; h,H = alpha helix.

Table 3. Putative antigenic domains in the E protein of yellow fever virus (17)

Antigenic domain	No. of aa (aa position)	Hydrophilicity per aa	Surface probability per aa	Flexibility per aa	Conformation		Antigenic index per aa
					CF	GOR	
1	6 (7–12)	0.62	3.10	1.00	h	—	0.72
2	6 (25–30)	0.90	5.13	1.02	h+t	T	1.00
3	7 (35–41)	0.74	5.32 (5)	1.01	T	T	1.01
4	7 (69–75)	1.11	4.31	1.03	T	T	1.50
5	13 (91–103)[a]	1.64	6.39 (4)	1.02	T	T	1.17
6	7 (129–135)	0.77	6.75 (2)	1.03	h	H	0.77
7	16 (145–160)	1.20	6.63 (3)	1.02	H	T	0.96
8	9 (203–211)	0.96	4.18	0.99	h	T	0.89
9	8 (223–230)	0.83	2.96	0.97	H	—	0.79
10	15 (263–277)	1.38	8.70 (1)	1.02	T	T	1.13
11	8 (294–301)	0.55	3.26	1.01	T+B	T+B	0.90
12	7 (308–315)	1.40	4.78	1.05	T	T	1.03
13	16 (380–395)[b]	1.42	5.76	1.03	t+B	T	1.00
14	7 (416–422)	0.39	1.04	1.13	t	—	0.58
15	8 (466–473)	0.67	3.86	0.98	B+t	—	0.78

[a]RGWG.
[b]GRGD.
CF = Chou-Fasman (27); GOR = Garnier-Osguthorpe-Robson (28); t,T = β-turn; b,B = β-sheet; h,H = alpha helix.

In Table 4 the 14 putative antigenic domains in D4V glycoprotein E are preserved. Once again putative antigenic domain #4 contains the amino acid sequence RGWG and putative antigenic domain #14 contains the amino acid sequence RGAK.

These analyses provide the basis for synthesizing peptides that may be able to induce antibody production in experimental animals. Roehrig et al. (11) used a computer program based on the computation of physical properties of the primary amino acid (aa) sequences (similar to that used in the present study) for flavivirus E glycoproteins to determine the putative antigenic domains in Murray Valley fever virus glycoprotein E. They reported that such synthetic peptides, designed according to the aa sequence of putative antigenic domains in the viral glycoprotein, elicited antibody responses in mice.

Amino acid sequence homologies between viral E glycoproteins

Tables 5 and 6 deal with the homologous amino acids in the E glycoproteins in the four flaviviruses analyzed in this study. It is of interest that the antigenic domain #4, which contains the RGWG sequence, is shared by the four viral glycoproteins (Tables 5 and 6A). It was also noted that an aa sequence VGR is found in JEV, WNV, and D4V, but not in YFV (Table 6B). Homologous aa sequences containing the aa GRGD are present in JEV, YFV, and WNV (GRGE) (Table 6C). D4V that did not contain the GRGD sequence has the sequence VRGA about 20 aa downstream (Table 6C). Hydrophobic aa sequences in the E glycoproteins situated prior to the membrane anchorage sequence are also homologous (Table 6D). The program GAP (20) was used to determine the overall similarity between the E glycoprotein of the four viruses. The putative antigenic domains in the four viral glycoproteins that contain the RGD-series sequences and homologous putative antigenic domains are presented in Table 7. Such domains may well be the common flavivirus antigenic domains.

Discussion

The strategic plan (during the period 1987–1992) in the World Health Organization program for dengue virus vaccine development (2) covers a number of approaches, including the development of synthetic peptides as part of the subunit vaccines approach. The computer analyses of the primary aa sequences of flavivirus E glycoproteins as presented in this study seem to be in agreement with the study of Roehrig et al. (11) on synthetic peptides from the E glycoprotein of dengue type 2 virus. In this study, computer modeling of the viral E glycoprotein was used to design 18 synthetic peptides comprising 80% of the extramembranal region. These linear peptides were found by Roehrig et al. (11) capable of immunizing BALB/c mice eliciting antiviral antibodies. Comparison of Roehrig's

Table 4. Putative antigenic domains in the E protein of D4V

Antigenic domain	No. of aa (aa position)	Hydrophilicity per aa	Surface probability per aa	Flexibility per aa	Conformation		Antigenic index per aa
					CF	GOR	
1	6 (7–12)	0.65	2.38	1.02	t+h	B	0.81
2	8 (44–51)	0.66	6.39	1.03	H	H	0.75
3	16 (77–92)	1.87	14.50 (1)	1.05	H+B	H+B	1.00
4	9 (95–103)[a]	1.02	2.53	1.02	t+T	B+T	1.22
5	5 (144–148)	1.11	1.77	0.81	T	—	1.06
6	8 (174–181)	0.58	3.28	1.02	T	T	1.02
7	13 (201–213)	0.85	14.02 (2)	0.99	B	H+T	0.86
8	14 (225–238)	1.29	8.78 (5)	1.00	B	H	0.83
9	9 (242–250)	1.19	9.89 (4)	1.01	h	H+T	0.90
10	8 (287–294)	0.87	10.38 (3)	0.99	h	H	0.66
11	13 (307–319)	1.04	4.61	0.93	H	H	0.86
12	7 (341–347)	0.74	6.70	1.01	h	H	0.53
13	7 (368–374)	0.95	6.15	1.02	h	B+t	0.97
14	9 (401–409)[b]	1.21	5.90	1.03	h+t	H	0.97

[a]RGWG.
[b]RGAK.
CF = Chou-Fasman (27); GOR = Garnier-Osguthorpe-Robson (28); t,T = β-turn; b,B = β-sheet; h,H = alpha helix.

Table 5. Putative antigenic domains in the virion glycoprotein of four flaviviruses

Antigenic domain	JEV aa	WNV aa	YFV aa	D4V aa	
1	6–12	5–12	7–12	7–12	
			25–30		
2	35–42	35–41	35–41	—	
			69–75	44–51	
3	75–88	78–87	—	77–92	
4 (RGWG)[a]	95–103	95–103	91–103	95–103	sequence homology
5	129–137	129–137	129–135	—	
6	—	—	145–160	144–148	
7	180–186	—	—	177–181	
8	190–197	—	—	—	
9	212–219	—	203–211	201–213	
10	225–238	227–234	223–230	225–238	
	—	237–245	263–277	242–250	
11	284–295	286–297	294–301	287–294	
12	303–317	—	308–315	307–319	sequence homology
	—	—	—	341–347	
13	374–380	—	—	368–374	homology
14 GRGD	387–403	385–397	380–395	—	
15 RGAK	—	—	—	401–409	
	—	407–412	416–422	—	
16[b]	473–479	470–477	466–473	—	

[a] Conserved sequence
[b] Intracytoplasmic

results to the present analysis of dengue type 4 virus E glycoprotein (Table 4) suggests that 5 of the 6 putative antigenic domains are similar to the antigenic peptides reported by Roehrig et al. (11).

Allan (9) studied the response of T-helper cells specific for flaviviruses and reported that synthetic peptides containing the amino acids (a) 6–15, (b) 157–167, (c) 248–258, and (d) 278–287 are recognized by T cells from mice immunized with whole infectious dengue type 2 virus. These T-cell epitopes correspond to the putative antigenic domains of dengue type 4 virus presented in Table 4. These differ from the antibody-inducing antigens described by Roehrig et al. (11). Dengue type 2 synthetic peptides, therefore, contain different antigenic epitopes, some responsible for inducing an antibody response and some involved in inducing T-cell proliferation. Computer analysis of dengue virus E glycoprotein has shown a remarkable capability to predict antigenic domains in glycoproteins of flaviviruses. Comparison of computer analyses with the experimental results obtained with synthetic antigenic peptides could provide an approach to using antigenic synthetic peptides of dengue virus E glycoprotein as vaccines. The main question is how such virus synthetic peptides could be effective in the immunization of humans in areas endemic for dengue viruses.

In considering the route of dengue vaccine application to humans, it is necessary to consider the natural route of infection. Monath (7) indicated that after a

Table 6. Amino acid homologies in five domains in the flavivirus E glycoproteins

A.

JEV	98	D	R	G	W	G	N	G	C	G	L	F	G	K	G	S	112
YFV	98	D	R	G	W	G	N	G	C	G	L	F	G	K	G	S	112
WNV	98	D	R	G	W	G	N	G	C	G	L	F	G	K	G	S	112
D4V	98	D	R	G	W	G	N	G	C	G	L	F	G	K	G	G	112
TBEV	98	D	R	G	W	G	N	H	C	G	L	F	G	K	G	S	112
Hydrophilicity			(+)							(−)							

B.

JEV	350	P	V	G	R	L	V	T	V	N	P	359
YFV	345	N	K	G	I	L	V	T	V	N	P	354
WNV	347	P	V	G	R	L	V	T	V	N	P	356
D4V	347	V	V	G	R	I	I	S	S	T	P	356
Hydrophilicity				(−)								

C.

JEV	376	P	P	F	G	D	S	Y	I	V	V	G	R	G	D	K	390
YFV	369	P	P	F	G	D	S	Y	I	I	V	G	R	G	D	S	383
WNV	373	P	P	F	G	D	S	Y	I	V	V	G	R	G	E	N	387
D4V	370	R	P	L	D	D	S	Y	I	V	I	G	V	G	N	S	384
												406	R	G	A	K	409
Hydrophilicity					(−)								(+)				

D.

JEV	421	G	D	T	A	W	D	F	G	S	I	G	G	V	F	N	S	I	G	K	439
YFV	414	G	D	T	A	W	D	F	S	S	A	G	G	F	F	T	S	V	G	K	432
WNV	418	G	D	T	A	W	D	F	G	S	V	G	G	V	F	T	S	V	G	K	436
D4V	415	G	E	T	A	W	D	F	G	S	V	G	G	L	F	T	S	L	G	K	434
Hydrophilicity										(−)											

Table 7. Putative common antigenic domains containing RG sequences in JEV, YFV, WNV, and D4V glycoprotein E

A.																										
JEV	93		K	Q	G	F	T	D	**R**	**G**	**W**	G	N	G	C	G	L	F	108							
				(−)				(+)						(−)												
YFV	92	C	K	R	T	Y	S	D	**R**	**G**	**W**	G	N	G	C	G	L	F	108							
							(+)										(−)									
WNV	92	C	K	Q	G	V	V	D	**R**	**G**	**W**	G	N	G	C	G	L	F	108							
			(−)							(+)						(−)										
D4V	92		C	R	D	V	V	D	**R**	**G**	**W**	G	N	G	C	G	L	F	108							
			(−)					(+)				(−)														
B.																										
JEV	384	V	V	**R**	G	**D**	K	Q	I	N	H	H	W	H	K	A	G	S	T	L	G	K	A	406		
			(−)										(+)									(−)				
YFV	378	V	G	**R**	G	**D**	S	R	L	T	Y	Q	W	H	K	E	G	S	S	I	G	K	398			
			(−)										(+)									(−)				
WNV	381		V	V	G	**R**	G	E	N	N	I	N	H	H	W	H	K	S	G	S	S	I	G	K	A	403
			(−)									(+)											(−)			
D4V	398	G	K	M	F	E	S	T	Y	**R**	G	A	K	R	M	A	412									

mosquito bite, dengue virus replicates in the skin at the site of the insect bite. One or two days later, the virus is present in the lymph nodes prior to the development of viremia. The nature of the cells in the skin in which dengue virus (and other flaviviruses) replicate after release from the infected insect is not known. Not much is known about the interaction of dengue virus with skin cellular elements such as keratinocytes and Langerhans cells.

The nature of the flavivirus receptors on cells, as well as the aa sequences in the virion E glycoprotein that interact with the cellular receptors, are not known. A possible aa sequence in glycoprotein E of flaviviruses that may interact with a cellular receptor is the RGD sequence in Japanese encephalitis virus and yellow fever virus E glycoproteins, RGE in West Nile fever virus, and RGA in dengue type 4 virus E glycoproteins. It should be noted that the aa sequence RGW is conserved in glycoprotein E of the four flaviviruses discussed in the present analysis, as well as in the E glycoprotein of tick-borne encephalitis (Table 6).

The sequence RGD was reported by Ruoslahti (29) to be the essential aa sequence in the extracellular protein fibronectin for its interaction with the cellular surface glycoprotein receptor integrin. The aa sequence RGD was reported to be conserved in foot-and-mouth disease virus isolates and is involved in the attachment of the virions to the cell surface integrin polypeptide receptor (14, 15). Since it was found that the synthetic peptide RGDS interferes with the adsorption of foot-and-mouth disease virions to their receptors on cultured cells (14, 15), it might be of interest to determine whether synthetic peptides like RGDS or RGW would interfere with the adsorption of flaviviruses like JEV and YFV to susceptible cells. Such information would be important for the development of synthetic peptides that might be able to interfere with virus infection of the skin. Such compounds would be incorporated in a lotion that enhances peptide penetration into the skin so as to be applied directly to the skin by people in dengue-virus endemic areas. Such an approach, if feasible, does not use an invasive method of treatment, the peptides have a long shelf-life, and their use does not require specially trained medical personnel.

Skin Langerhans cells function by engulfing sensitizing antigens in the epidermis and are known to take up and present the antigens, e.g., simple haptens, metals, proteins, or viruses, to T cells (reviewed in 4). Hence, it may also be possible that Langerhans cells will present synthetic antigenic peptides that are introduced into the skin (e.g., with dimethylsulfoxide) to the immune system. If virus receptors on skin dendritic cells are able to interact with the synthetic peptides RGD, RGE, or RGA, these receptors would be rendered nonfunctional for virus attachment. In addition, introduction into the skin of synthetic peptides containing antigenic domains that are not involved in aggravating the disease may cause a local antiviral immune response in the skin and in the lymph nodes. Such an approach might allow elimination of antibodies to viral proteins that enhance the disease. Since one of the major problems in immunization against dengue is the development of antibodies that enhance the disease, it is hoped that suitable synthetic viral antigenic epitopes responsible for virus neutralization could be selected that will ensure protection against dengue.

Two new aspects for the development of dengue vaccines have been discussed in the present study that might complement the reported WHO strategy: a) the need to understand the events in virus infection in the skin at the site of the insect bite, with the emphasis on the interaction of flaviviruses with skin Langerhans cells and b) the utilization of synthetic peptides to prevent virus-cell interactions in the skin. A novel approach to protect populations against flavivirus infections would be the use of synthetic peptides applied directly to the skin to interfere with virus infection. Utilizing the skin for enhancing immunity against flavivirus infections might decrease the cost of treatment and eliminate the need for injection of vaccines.

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