Antigenicity of flaviviruses

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Summary. The importance of viral structural and non-structural antigens in flavivirus pathogenesis has not been satisfactorily assessed. This review examines the antigenic interrelationships of the flaviviruses, considers some of the implications of antigenic cross reactivity in virus virulence and highlights potential consequences of virus-antibody interactions.

Introduction

Viruses in the family *Flaviviridae*, of which yellow fever is the type species [94], are responsible for considerable human morbidity and mortality in particular areas of the world. There are currently 68 registered antigenically related, but distinct, flaviviruses, some of which are known to produce diseases causing uncomplicated febrile illness, perhaps accompanied by a rash, to hemorrhagic fever, shock syndrome, hepatitis or encephalitis (for reviews see [67, 57]). The morphological appearance of a single virion is illustrated schematically in Fig. 1. The particles are spherical with a diameter of 40–50 nm. There are three structural proteins: the capsid (C; M_r 13,000 to 16,000), which is surrounded by a host-derived membrane incorporating 2 viral proteins; the membrane protein (M; M_r 8,000), derived from a glycosylated precursor (prM; M_r 24,000 to 27,000), and the envelope (E; M_r 50,000 to 59,000), which is usually glycosylated. It is not yet clear if the C-terminal of the E protein consists of dual transmembrane elements, shown as a hooked structure [54, 91], or if there is a single transmembrane structure. This latter proposal derives from the observations of Aaskov et al. [2] and Innis et al. [48] who detected strong serological responses to the first hydrophobic transmembrane region, suggesting it could be exposed on the surface of the lipid envelope (both dual and single transmembrane elements are depicted in Fig. 1). The flaviviruses have a linear positive-stranded

Fig. 1. Schematic representation of a flavivirus particle. Two forms of the virion are depicted; on the right, the virion has a small transmembranal span of appropriate polypeptide whereas on the left, the polypeptide is shown as a single transmembranal span; A viral RNA; B viral capsid; C lipid membrane; D membrane protein; E envelope protein

infectious RNA molecule between 10,000 and 11,000 nucleotides in length. The genomes of several flaviviruses have been sequenced and they have a similar organization [18, 24–26, 53, 54, 83, 85, 87]. The viral proteins are encoded in one open reading frame which represents about 90% of the genome. Seven non-structural virus-coded proteins are recognized and the entire gene order of both the structural and non-structural proteins is now known to be C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5 [4, 5, 7, 19, 69, 70, 80].

The flaviviruses, originally defined as the group B arboviruses, were shown to cross-react in standard serological tests [17]. They were subsequently rearranged into 7 serogroups on the basis of their antigenic interrelationships in plaque reduction neutralization tests and also by taking into account the invertebrate vector, where recognized [65]. Additional viruses were recently examined in neutralization tests and the sub-groupings were updated accordingly [11].

With the advent of monoclonal antibody technology it became possible to examine these antigenic interrelationships in considerably more detail than was previously possible. Monoclonal antibodies (MAb) have therefore begun to be used in studies of flavivirus proteins. These studies contribute data on the possible significance of viral protein interactions with antibodies in flavivirus pathogenesis.

The viral envelope glycoprotein

Prior to the use of MAb it was recognized that the E glycoprotein of flaviviruses contained at least three classes of antigens, viz. type specific, complex reactive and flavivirus group reactive [16, 22, 86]. Subsequently, there have been several extensive analyses of antigenic cross reactivity between flaviviruses, particularly with E-specific MAb [28, 30, 33, 43, 44, 50, 51, 58, 63, 74, 82]. Antibody binding assays identify reactivity in non-functional tests and these have confirmed and extended the original observations. Some typical results obtained using indirect immunofluorescence tests with E-specific MAb and a large number of flaviviruses are presented in Table 1. Unique epitopes can distinguish between individual virus strains (e.g., 864), some epitopes are virus-specific (e.g., 825, 546 or 995), whilst others show intermediate reactivity with other flaviviruses (e.g., 126), particularly within individual serogroups. Finally, broadly cross reactive epitopes (e.g., 813) confirm the common ancestral origin of the flaviviruses. It is of

Table 1. Antigenic cross reactivity analyses between flaviviruses, using monoclonal antibodies (against serogroup 3 viruses) that identify the viral E protein

MAb	56 Flaviviruses (group arranged)															
	GLLMNPRSCT	ACCDEJKKMMPRSS	AABIJJKKMNSSSUWVY	SZ	BINT	BBERU	DDDD									
	GGIEEOFREY	PBRBNUAOOMPBAB	LRSLBUOUVJLETSSNF	PI	ATTM	AOHOG	EEEE									
	YT AGW EEU	OC TTDUDLB BK	FOQHEGKNENEPRL	OK	G AU	NU CS	NNNN									
		I	A	A			1234									
864							+									
825							+									
411							+									
117							+									
546							+									
995			+													
541	+						+	+								
127			+				+	+								
538				+			+			+						
549			+				+			++						
427	+		+				+									
623				+	+		+	+								
140	+		+				+			++						
101/R3		+	+				+++ ++	+		++ +						
F6/16A	+	+	+				++ +	+		++ +					+	
126		+	+	+	+	+	++ ++ ++	+	++	++ + ++ +						
38	++ ++ +	+	+	++	+	++ ++++++	++ +++++	+	++ +	++ + +						
39	+++ ++ +	+	++	+	++ ++++++	++ +++++	++ +++++	+	++ +	++ + ++						
110	++ ++ ++	++ + ++	++++	+	+++ ++	++ +++++	++ +++++	++	++ +	++ +						
810	+++++	++ ++++++	++	+	++++	+++++	+++++	++	+	++ + +++++						
868	++ ++ ++	++ ++++++	+	++	++++	++ +++++	++ +++++	+	++ +	++ + +						
843	++ ++ ++	+++++	++	++	++++	++ +++++	++ +++++	+	++ +	++++ +						
528	+++++	++++	++++	++	++++	++++	++++	++	++ +	++++						
612	++ ++ ++	+++++	+	++++	++++	++++	++++	++	++ +	++++						
T7	+++++	+++++	++++	+	++++	++++	++++	++	++ +	++++						
813	+++++	+++++	++++	+	++++	++++	++++	++	++ +	++++						
F7/3	++++	++++	++++	+	++++	++++	++++	++	++ +	++++						
RH1	++++	++++	++++	+	++++	++++	++++	++	++ +	++++						
RH2	++++	++++	++++	+	++++	++++	++++	++	++ +	++++						

interest that viruses within the tick-borne complex tend to exhibit relatively low levels of cross reactivity with viruses outside this complex. On the other hand, viruses in serogroup 3 tend to share E-specific epitopes with viruses in other serogroups. This is supported by the evidence already presented in Table 1 and further typified by results (Table 2) which show the relatively limited extent of cross reactivity with other flaviviruses when MAb prepared against the tick-borne virus louping ill, were analysed. Thus, MAb analyses of cross reactivities among flaviviruses have revealed both conserved and non-conserved epitopes in the envelope glycoprotein and exposed relationships that were not previously apparent. Such tests also demonstrate the usefulness of these antibodies to identify closely related viruses for diagnosis and in general support the serogroupings suggested by Porterfield [65].

Plaque reduction neutralization tests (functional analyses) with E-specific MAb reveal entirely different antigenic cross relationships from those seen in antibody binding tests. In general, MAb that are cross reactive in binding analyses show specificity for closely related flaviviruses in neutralization tests [9]. However, heterologous cross reactivity in neutralization tests has been seen. For example, a MAb derived using Japanese encephalitis virus neutralized several flaviviruses but failed to neutralize Japanese encephalitis virus. Similar heterogeneity in the neutralization test was also reported by Roehrig et al. [72] with St. Louis encephalitis, by Kimura-Kuroda and Yasui [50] with Japanese encephalitis, St. Louis encephalitis, West Nile and Murray Valley encephalitis, and by Yasui [95] with a dengue virus. It is also of interest that both de Madrid and Porterfield [27] and Calisher et al. [11] reported that yellow fever virus is not neutralized by polyclonal antisera

Table 2. Indirect immunofluorescence antigenic cross reactivity analyses between flaviviruses, using monoclonal antibodies against LI virus

MAb	56 Flaviviruses (group arranged)															
	GLLMNPRSC	ACCDEJ	KKMM	PRSS	ABIJJ	KKMN	SSSU	WWY	SZ	BINT	BBERU	DDDD				
	GGIEEOFREY	PPRBN	UA	OOMP	BAO	RSLBU	OUVJ	LETSS	NF	PI	ATTM	AOHOG	EEEE			
	YT AGW EEU	OC	TTDUD	LB	BK	OQHEG	KNELE	PRUL		OK G	AU	NU	CS	NNNN	1234	
		I				A				A						
35.13a	+	+	+	+	+			+								
35.7		+	+		+					+	+		+			
35.6a	+	+		+	+					+	+		++			
35.10b	+	+	++	+	+			+		+						
35.12a	+	+	+	+	+	+										
35.1a	+	+	+	+	+	+	+	+			+					
35.5b	+	+		+		+	+	+		+	+		+			
35.3a	++	++	+		+	+	+						+			+
35.4a	+	+	++	+	+	+		+		+					+	+
35.14b	+	+	+	+++	+	+				+	+		+			+
35.11b	+	+		+	+	+	+	+		+	+		+			
34.2	+++++	+++++	+	+	++	+	+	+	+						++	+
34.1	+++++	+++++	++	++	+	+	+	+	+				+		++++	

against other flaviviruses and that antiserum against yellow fever virus does not neutralize other flaviviruses. For this reason, yellow fever virus was not assigned to a serogroup in the most recent antigenic analysis of the flaviviruses [11]. The results presented by Buckley and Gould [9], with hyperimmune antisera, seem to contradict this observation since the hyperimmune antisera that were produced against either yellow fever or West Nile virus (RH1 and RH2 respectively) were positive in neutralization tests with both yellow fever and other flaviviruses. This difference in performance of antisera highlights one of the problems of using functional tests to classify viruses and almost certainly reflects the different methods used for production of the polyclonal hyperimmune antisera. In addition, neutralization of flavivirus infectivity depends not only upon the presence of the appropriate epitope on the envelope glycoprotein but also upon its precise presentation [9]. Different yellow fever strains showed significant differences in their readiness to be neutralized by known monoclonal and polyclonal neutralizing antibodies. These observations demonstrate the importance of selecting the most suitable virus strain if the test is to be used as the basis for virus classification.

When administered passively, some antibodies against the viral E protein also have the capacity to protect animals against challenge with virulent virus. However, antibodies that show neutralization *in vitro* may or may not show protection *in vivo* against challenge virus. Furthermore, non-neutralizing E-specific antibodies may show protection [8, 9, 20, 34, 42, 45, 56]. In some cases, protection *in vivo* is probably mediated directly via neutralization of virus infectivity. In other cases, different mechanisms probably operate to protect the host. For example, virus aggregation would be expected to reduce virus infectivity. On the other hand complement-mediated cytotoxicity at the infected cell surface, could kill the infected cells, leading to protection of the infected host. In either situation the host would gain time to mount a successful immune response. Heterologous cross protection has also been observed in which MAb against either Japanese encephalitis or yellow fever were found to protect mice against challenge with other flaviviruses (unpubl. results). However, as was observed with neutralization, antibodies that protect against one strain of virus may fail to protect against another strain of the same virus, even though the antibody binds the different strains of virus with the same avidity [12]. The success or failure of E-specific MAb to protect mice against challenge with different strains of yellow fever virus was found to be partly dependent upon the inherent virulence of the virus strain, as measured by the time taken to kill mice. For example, the FNV strain of yellow fever virus killed weanling mice within 7 days [3] and we have consistently failed to identify MAb that can passively protect mice against this strain. However, if a strain of yellow fever that takes a longer time to kill mice was used, the passively administered E-specific MAb could protect mice. Protection in these cases is probably due to a

combined effect of the passively administered antibody and the natural host immune response.

Monoclonal antibodies characterized as above, have been used in solid phase competition binding ELISA tests to map the topological arrangement of the antigenic determinants in the E glycoprotein of several flaviviruses [12, 20, 42, 46, 90]. Several facts emerge from these types of studies. The glycoprotein spike consists of at least 3 antigenic domains, each of which contains epitopes that haemagglutinate and/or induce antibodies responsible for neutralization of virus infectivity, protection of mice against virus challenge and enhancement of virus infectivity via Fc-receptor bearing cells. Epitopes that are conserved throughout the flaviviruses, map together and tend to exhibit poor neutralization activity, whereas non-conserved epitopes map in distinct regions and, in general, exhibit potent neutralizing activity. Some epitopes appear mobile in the sense that they may occupy different relative locations in different strains of the same virus. Finally, antibody binding to flavivirus E protein epitopes may induce conformational changes of the surface glycoprotein, revealed by enhanced antibody binding [43].

On the basis of nucleotide and amino acid sequence data, together with knowledge of the structural characteristics, assignment of disulphide bridges, epitope topology and studies with synthetic peptides, structural models of the E protein of West Nile, tick-borne encephalitis and Murray Valley encephalitis have been created [55, 60, 71]. These models predicted the existence of three antigenic domains in the primary structure, localised individual epitopes in the glycoprotein, identified 2 hydrophobic sequences that have properties typical of membrane anchor regions, revealed potential T-cell determinants conserved in all flaviviruses and revealed features of the folding of the polypeptide chain, including the generation of discontinuous protein domains. Future crystallographic analysis of this protein should elucidate the structure of the receptor site and the domain(s) for virus neutralization.

Non-structural flavivirus proteins

Extensive cross reactivity binding analyses also have been carried out using MAb specific for the NS1 protein. These MAb characteristically show only very limited cross reactivity with heterologous flaviviruses (Table 3). Studies with MAb against non-structural flavivirus-coded proteins, perhaps surprisingly, have revealed functional activity. Schlesinger et al. [75] and Gould et al. [34] reported protection of mice against virulent yellow fever virus, if the mice were given MAb against the NS1 protein of yellow fever virus immediately prior to virus challenge. Active immunization of mice and monkeys against yellow fever virus infection has been accomplished using either purified NS1 protein [76] or NS1 β -galactosidase fusion protein [13].

Table 3. Indirect immunofluorescence antigenic cross reactivity analyses between flaviviruses, using monoclonal antibodies against the NS1 protein of serogroup 3 viruses

MAb	55 Flaviviruses (group arranged)											
	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
	GLLMNPRSCT	ACCDEJKKMMPRSS	ABIJJKKMNSSSUWWY	SZ	BINT	BBERU	DDDD					
	GGIEEOFREY	PPRBNUAOMPBAO	LSLBUOUVJLETSSNF	PI	ATTM	AOHOG	EEEE					
	YT AGW EEU	OC TTDUDLB BK	FQHEGKNELEPRUL	OK	G AU	NU CS	NNNN					
		I		A			1234					
863				+								
871				+								
979				+								
429				+								
423				+		+						
993			+	+								
999			+	+								
992	+		+	+								
917			+	+	+							
924			+	+	+						+	
925			+	+		+						
615			+	+								
618			+	+								
492		+	+	+								
428	+			+								
109	++	+	+	+		+						

The NS1 protein is thought to be associated with the plasma membrane of infected cells [14, 34] and some antibodies to NS1 have shown cytolytic activity [75]. Protection might therefore result from lysis of infected cells bearing NS1 antigen.

The prM protein of most flaviviruses is considered to be a non-structural protein [77, 92]. However, both polyclonal antisera and MAb against dengue-1 virus prM bind to purified virions [1, 49] and at least one prM-specific MAb induces antibody dependent enhancement of infectivity in Fc-receptor bearing cells [46]. Therefore for dengue viruses at least, the prM protein is probably structural. In contrast with E-specific MAb, those that identify dengue prM protein do not appear to show cross reactivity in binding analyses with any other flaviviruses [44]. Nevertheless, functional activity with these MAb was recently observed [49]. These authors found that antibodies against dengue prM, administered passively, can protect mice against challenge with both homologous and heterologous dengue serotypes. Some prM-specific MAb also show low titer virus neutralization. The mechanisms of protection and neutralization are not clear but steric hindrance of E protein epitopes by added prM antibodies or antibody mediated cytolytic activity, probably in the presence of complement, seem to offer the most likely explanation for neutralization and protection by prM antibodies. Whether or not the prM protein has significance in the immunopathology of dengue haemorrhagic fever and dengue shock syndrome remains to be determined.

There are relatively few antigenic studies of flavivirus proteins other than those referred to above; however, a MAb which identified the non-structural p74 protein (NS3) of Japanese encephalitis virus showed antigenic cross reactivity with flaviviruses from several antigenic complexes [32]. This MAb was unusual since it also identified an epitope present in the nucleus of vertebrate cells. Such epitope mimicry might have significance in viral immunopathology.

Antibodies and pathogenesis

In addition, to neutralization of virus infectivity *in vitro*, antibodies specific for E protein may also enhance the susceptibility of Fc-receptor bearing cells to infection by viruses [36, 41] and this phenomenon is usually referred to as antibody dependent enhancement (ADE) of virus infectivity (for reviews, see [67]). With flaviviruses, ADE occurs in the presence of sub-neutralizing antibody concentrations when peripheral human blood monocytes or murine and human macrophage-like cell lines are used; these cells bear Fc-receptors [20, 21, 37, 47, 61, 64, 66, 73]. Enhanced virus infectivity is due to an increased number of infected cells in the presence of antibody complexed with virus [62]. Non-neutralizing antibodies may also elicit ADE [21]. Binding of the antibody-virus complexes to the Fc and/or complement receptors [15] leads to internalization and thus infection, by the non-neutralized virus, of the receptor bearing cells.

There is some evidence that an equivalent immune-mediated phenomenon exists *in vivo* with flaviviruses. Halstead [38] proposed that ADE was an important factor for the development of dengue haemorrhagic fever and dengue shock syndrome in the course of sequential infections of children possessing maternally acquired antibodies. There are no reports of a similar situation in nature with flaviviruses other than the dengue serotypes. However, an equivalent phenomenon with some other viruses has been seen. For example, "early death syndrome" after rabies virus infection in inadequately immunized humans or animals has been reported [6, 68, 79] and a similar early death effect was reported in kittens inoculated with feline infectious peritonitis virus following passive transfer of antiviral antibody [89].

Following the observations of Webb et al. [88], that increased viraemia occurred in mice challenged with Langat virus in the presence of antibody, attempts to develop an *in vivo* model with which to study virulence enhancement have met with some success using yellow fever and Japanese encephalitis viruses [31]. It was initially found that yellow fever E-specific MAb which do not protect mice against infection with yellow fever virus can shorten the average survival time of mice challenged with virulent yellow fever virus [3]. Subsequently, antibodies that could protect mice were shown to increase virus virulence if they were administered intraperitoneally after

the virus had become established in the target tissue [35]. Detailed analysis of the growth characteristics of the viruses in mice immunocompromised with hydrocortisone, cobra venom or anti-thymocyte serum demonstrated that enhanced virulence probably results from the induction by virus-antibody complexes of an Arthus type 3 hypersensitivity reaction [35]. A similar observation has now been made with louping ill virus using louping ill virus-specific MAb (H. Reid, pers. comm., 1989). Whether or not antigenic cross-reactivity between flaviviruses has significance in the immunopathology of flavivirus disease characteristics remains to be determined. However, it may be significant that severe cases of human Japanese encephalitis infection often show high IgG antibody responses, implying an anamnestic response, whereas the less severe cases show low IgG but a high IgM response, implying a primary immune response (A. Mathur, pers. comm., 1986).

Studies of the replication cycle of flaviviruses have mostly shown that virus proteins occur only in the cytoplasm of infected cells. Unequivocal evidence that flavivirus-specific proteins can be found within the nuclei of flavivirus infected cells has only recently been forthcoming. Its significance in virus replication and/or pathogenesis has not yet been identified. Previous reports of dense particles in the nuclei of cells infected with flaviviruses [59, 85, 96] had not been confirmed and evidence of a virus-specific step in the nucleus was unconvincing [93]. Recently, Buckley and Gould [10] identified 2 MAb, prepared against yellow fever or West Nile virus, which produced fluorescent labelling of cytoplasmic antigen in cells infected with the virus used to derive the antibodies. However, when these antibodies were tested with Langat (for the West Nile derived virus) or Zika virus infected cells (for the yellow fever derived virus), they produced virus-specific nuclear as well as cytoplasmic fluorescence. Independently, Tadano et al. [84] also observed virus-specific nuclear fluorescence in dengue-4-infected cells tested with a core protein-specific MAb derived from dengue-4 virus. Further support for a role of the nucleus in the replication of flaviviruses comes from recent evidence obtained using MAb prepared against the French viscerotropic strain of yellow fever virus (yellow fever FVV). Many of these MAb produced very distinct nuclear fluorescent labelling of yellow fever-infected cells (Fig. 2). The presence of the same antigen at the peripheral part of the cytoplasm, on the plasma membrane, is also evident. Double fluorescence labelling of the infected cells using a nuclear and a cytoplasmic antibody confirmed that the nuclear fluorescence was present only in yellow fever-infected cells (data not shown).

In addition to viruses sharing common antigenic determinants with each other, they may also share antigenic determinants with the host cells that they infect [23, 29, 32, 39, 40, 52, 78]. This is often referred to as molecular mimicry. Currently, there is no direct evidence that flavivirus infections can be rendered more severe due to autoimmune responses. However, molecular

Fig. 2. Indirect immunofluorescence microscopy of cells infected with yellow fever virus and labelled with a monoclonal antibody that produces both nuclear and cytoplasmic fluorescence of infected cells. Note how the fluorescence appears in the plasma membrane as well as completely filling the nuclei

mimicry has been observed with Japanese encephalitis virus epitopes [32, 81] and human sera taken from Japanese encephalitis-infected patients with encephalitis contained anti-nuclear antibodies with the same fluorescence characteristics as the Japanese encephalitis MAb that showed anti-nuclear fluorescence [32]. In addition, a MAb against louping ill virus which identified both the viral envelope glycoprotein and a cellular nuclear epitope produced enhanced virulence of louping ill virus when given passively to mice prior to challenge with the virus (Gould, unpubl. results). Finally, anticellular antibodies, that do not react with the immunizing virus, are frequently detected in the course of deriving and identifying virus-specific MAb. On several occasions we have observed enhanced virus virulence when such anticellular antibodies are used in antibody mediated enhancement experiments with flaviviruses.

Passive transfer to mice of virus-specific anticellular antibodies mimics, to some extent, the development of an autoimmune response. We are beginning to accumulate evidence that these types of antibodies may alter disease severity at least under experimental conditions. Epitope mimicry amongst viral and cellular determinants is not uncommon, particularly with the flaviviruses [81] and such mimicry could lead to situations in which viral

infections trigger an autoimmune response and thus aggravate the severity of the infection.

The antigenic cross reactivity of flaviviruses is evidently very complex and in many examples, the consequences of these antigenic interrelationships have not been assessed. It seems improbable that amongst such a large family of closely related viruses, the 4 dengue serotypes should be the only ones to exhibit enhanced immunopathology resulting from shared antigenic determinants. There is still considerable scope for studies of the antigenic characteristics of the flaviviruses particularly with respect to the immune response of the host.

Note added in proof. The entire nucleotide sequence of the envelope glycoprotein of louping ill virus has now been determined (Shiu, Ayres, and Gould, manuscript in prep.). The results show that louping ill virus shares a greater degree of homology with the western sub-type of tick-borne encephalitis virus than with the eastern sub-type and that both tick-borne sub-types are more closely related to louping ill virus than any of the known mosquito-transmitted flaviviruses.

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