J. gen. Virol. (1974), **23**, 91–96
Printed in Great Britain

The Flaviviruses (Group B Arboviruses): a Cross-neutralization Study

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(*Accepted* 13 *December* 1973)

SUMMARY

Cross-neutralization studies on 42 flaviviruses and their respective antisera were performed by a plaque assay in a line of pig kidney cells. Six tick-borne viruses fell into one subgroup; seven viruses associated with bats and small rodents and a further tick-borne virus fell into a second subgroup. Twenty-two mosquito-borne viruses fell into one major and four minor subgroups. Four mosquito-borne, one tick-borne and one bat virus showed no relationship to any other flavivirus.

INTRODUCTION

More than 40 distinct flaviviruses, or group B arboviruses, are now recognized, and the genus comprises the larger of the two now established within the family of Togaviridae, the smaller genus being the alphaviruses, or group A arboviruses. The genus derives its name from the type species, yellow fever virus, which is transmitted by mosquitoes, but some flaviviruses are transmitted by ticks, and others appear to spread without an arthropod vector. The development of a simple micro-method for the titration of flaviviruses in a cell culture system (Madrid & Porterfield, 1969) made possible a comprehensive study of antigenic relationships between different members of the genus as determined by cross-neutralization tests. The results confirm the separate identity of the tick-borne viruses as a sub-group within the genus, readily distinguishable from the mosquito-borne viruses. They also reveal a further sub-group of flaviviruses which are neither tick-borne or mosquito-borne, but are associated in nature with bats and small rodents.

METHODS

These are fully described elsewhere (Madrid & Porterfield, 1969). Essentially the culture system uses a cloned line of porcine kidney cells (PS cells) grown in air in Leibovitz L15 medium (Leibovitz, 1963) supplemented with 10 % tryptose phosphate broth and 3 % foetal or new-born calf serum. Immune sera were prepared in rabbits of the New Zealand white strain using one of four different regimens. Antigens were made from 10 % (w/v) suspensions of infected mouse brain in 0·9 % NaCl which was centrifuged at 10000 rev/min for 1 h (6500 *g*). Rabbits received one intravenous inoculation of 1 ml of antigen, or three intravenous inoculations at 7 day intervals, or five intravenous inoculations at daily intervals, or a single intramuscular inoculation with Freund's complete adjuvant; bleedings were made at 7, 14, 21 and 28 days, and the 28 day samples were used as a routine. An antiserum was accepted as satisfactory if it gave an antibody titre of 1:40 or greater against the

Table 1. *Results of cross-neutralization tests with 42 flaviviruses and their respective antisera*

Virus and abbreviation		Antiserum and immunization course																								
		NEG 1	LGT 3	KFD 3	LI 5	OHF Ad	TBE Ad	KDM 3	APO 3	DB 1	EB 3	BB 1	RB 3	MOD 1	CR 1	JBE 3	MVE 1	WN 3	SLE 5	KUN 1	USU 1	KOK 1	STR 5	ALF 3	SPO Ad
Negishi	NEG	**4**				2	4																			
Langat	LGT		**7**				6																			
Kyasanur Forest	KFD		2	**7**																						
Louping ill	LI				**3**	2	3																			
Omsk HF	OHF					**5**	3																			
Tick-borne E	TBE		2			2	**5**																			
Kadam	KDM							**7**																		
Apoi	APO	2	1					2	**7**	2			2													
Dakar bat	DB									**4**		3														
Entebbe bat	EB							2		2	**7**	3	2	1		4		3								
Bukalasa bat	BB									1		**5**		1		2										
Rio Bravo	RB									2		2	**5**	2		2	2									
Modoc	MOD													**7**	3											
Cowbone Ridge	CR													3	**7**											
Japanese BE	JBE															**5**	6									
Murray Valley E	MVE															2	**3**	5	3		5					
West Nile	WN															2	2	**7**	2	2	2					
St Louis E	SLE															3	1	7	**7**							2
Kunjin	KUN															2		7		**5**						
Usutu	USU															4	3	3			**2**					
Kokobera	KOK																3					**7**	4			
Stratford	STR																						**2**			
Alfuy	ALF																3				3			**2**		

Table 1 continued on next page.

homologous virus. Multiple doses of antigen were used (or adjuvant) only when single dose antisera were inadequate. Antisera were inactivated at 56 °C for 30 min. Details of the immunization regimens used with individual viruses are presented in Table 1.

RESULTS

Table 1 presents the results of testing 42 different viruses against 42 different antisera. Each antibody activity represents the mean of at least two titrations. In practice, every serum was first screened at a dilution of 1:20 against one virus, using two wells per serum, except for the homologous serum which was tested over a range of dilutions from 1:20 to 1:1280. Any serum which neutralized or partially neutralized a virus in the screening test was re-tested over a range of dilutions. Fig. 1 shows a screening test with Negishi virus. The homologous antiserum (H) gave complete neutralization and two others, Omsk haemorrhagic fever virus antiserum (O) and tick-borne encephalitis virus antiserum (TB) gave partial

Table 1. (*cont.*)

		SPO Ad	ZIK 1	CHU 1	IT 3	NTA 3	TMU 1	BAN 1	UGS Ad	EH 3	D 3	D2 5	D3 Ad	D4 Ad	YF 3	BSQ 3	WSL Ad	ILH 3	MML 1	POW Ad
Spondweni	SPO	**4**		6																
Zika	ZIK	3	**7**																	
Chuku	CHU	5		**7**																
Israel Turkey	IT				**4**		2													
Ntaya	NTA				6	**6**	3													
Tembusu	TMU				1		**7**													
Banzi	BAN							**5**	5											
Uganda S	UGS							5	**7**											
Edge Hill	EH								2	**3**										
Dengue 1	D1										**6**									
Dengue 2	D2										3	**5**	5							
Dengue 3	D3												**4**	2						
Dengue 4	D4												4	**3**						
Yellow fever	YF														**7**					
Bussuquara	BSQ															**5**				
Wesselsbron	WSL																**6**			
Ilheus	ILH																	**5**		
Montana ML	MML																		**5**	
Powassan	POW																			**6**

The number following each antiserum represents the immunization schedule used for that particular antiserum: 1 = 1 dose of antigen intravenously; 3 = 3 doses of antigen intravenously on days 1, 8 and 15; 5 = 5 doses of antigen intravenously on days 1, 2, 3, 4 and 5. Ad. = 1 dose of antigen intramuscularly with Freund's adjuvant. The numbers represent activities in neutralization as the reciprocals of antibody titres. Thus final serum dilutions of 1:20, 1:40, ..., 1:1280 are shown as 1, 2, ..., 7. Homologous reactions are in bold type. All cross-reactions not numbered showed no neutralization at 1:20 serum dilution.

neutralization. When retested in dilutions, the titre of the homologous serum was 1:160 (= 4 in Table 1), as was the titre with TBE antiserum, whilst the Omsk antiserum gave a titre of 1:40 (= 2 in Table 1).

As shown in Table 1, the flaviviruses can be divided into the following subgroups:

Subgroup 1. This contains six viruses, Negishi, Langat, Kyasanur Forest, Louping ill, Omsk haemorrhagic fever and tick-borne encephalitis viruses. All of these six viruses were isolated from ticks, as were the two other flaviviruses, Powassan and Kadam. In our tests, Powassan virus was unrelated to any other flavivirus, but Kadam virus fell within the second subgroup.

Subgroup 2. This contains eight viruses: Kadam, Apoi, Dakar Bat, Entebbe bat, Bukalasa Bat, Rio Bravo, Modoc and Cowbone Ridge. Kadam virus was isolated in Kenya from ticks collected from cattle. Apoi, Modoc and Cowbone Ridge viruses were isolated from rodents, the first in Japan and the other two in the U.S.A. The remaining four viruses were all isolated from bats. Montana myotis leuco-encephalitis virus was also isolated from bats, but is unrelated to the other viruses in subgroup 2, or indeed to any other flavivirus in our tests.

The remaining 26 viruses, excluding subgroups 1 and 2, Powassan and Montana ML

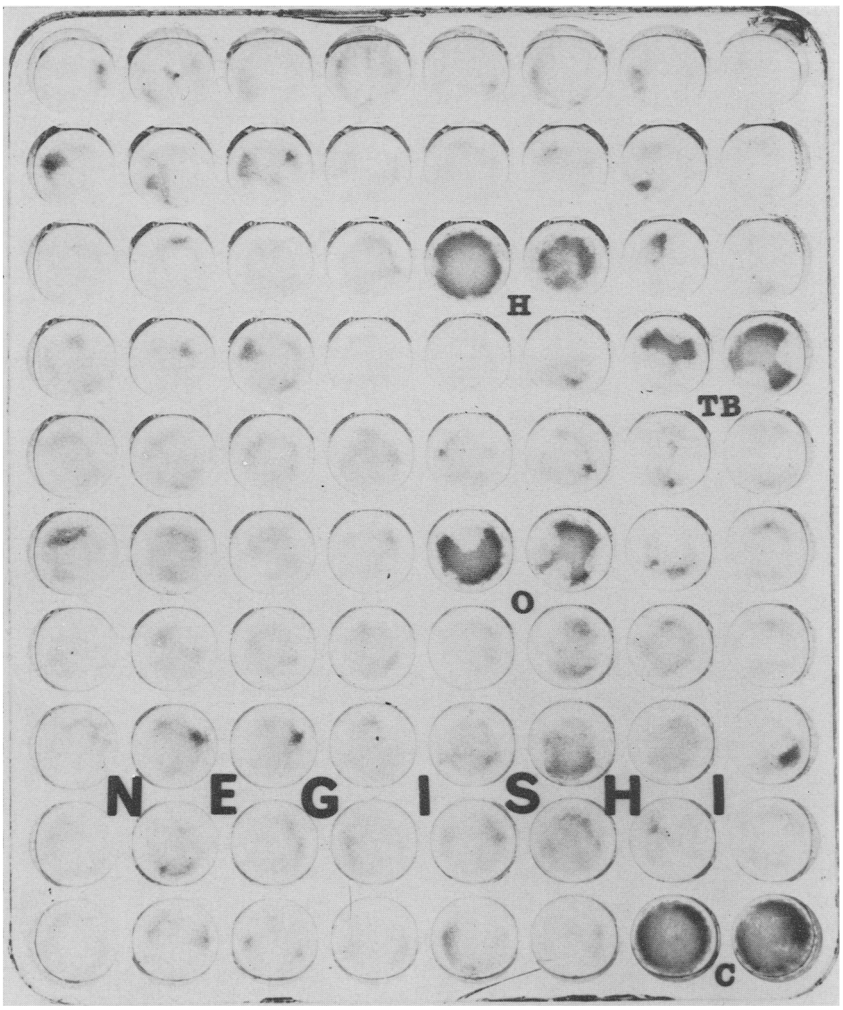

Fig. 1. Screening test with Negishi virus against 39 antisera each at 1/20 dilution overall, two wells per antiserum. C, Cell control; H, homologous antiserum; O, Omsk antiserum; TB, tick-borne encephalitis antiserum. All the other sera show no neutralization of the infectivity of Negishi virus present at about 50 p.f.u./well.

viruses, are all mosquito-borne in nature. By neutralization tests they fall into the following subgroups:

Subgroup 3. This contains nine viruses: Japanese encephalitis, Murray Valley encephalitis, West Nile, St Louis encephalitis, Kunjin, Usutu, Kokobera, Stratford and Alfuy viruses.

Subgroup 4. This contains three viruses: Spondweni, Zika and Chuku.

Subgroup 5. This contains three viruses: Israel turkey, Ntaya and Tembusu.

Subgroup 6. This contains three viruses: Banzi, Uganda S and Edge Hill.

Subgroup 7. This contains the four Dengue type viruses.

Six viruses show no relationship to any other flavivirus by our tests; these are yellow fever virus, Bussuquara, Wesselsbron and Ilheus viruses, in addition to Powassan and Montana ML viruses, already referred to with respect to subgroups 1 and 2.

DISCUSSION

The flavivirus genus comprises viruses which share certain basic physical and chemical properties and are distinguished from each other by serological tests. When the concept of arbovirus groups was first put forward, it was recognized that the haemagglutination-inhibition test showed the least resolution of antigenic differences, the neutralization test was the most specific, and the complement-fixation test was intermediate between these two (Casals, 1957). The present findings are valid for one set of sera tested under conditions which are standardized with respect to some parameters, and it is likely that another set of sera would yield different results. When the same sera were examined by haemagglutination-inhibition tests, many apparent cross-reactions were found (Madrid, 1972). These cross-reactions in haemagglutination-inhibition tests showed no clear pattern although there were statistically significant associations between the reactions obtained with the six viruses, Langat, Kyasanur Forest disease, Louping ill, Omsk haemorrhagic fever, tick-borne encephalitis and Powassan. The neutralization test results in this study bring in Negishi virus, another tick-borne virus, but they exclude Powassan virus. In their tabulation of the flaviviruses, Theiler & Downs (1973) list eight tick-borne viruses, the above named (including Powassan and Negishi) and Kadam virus.

To our knowledge, the serological delineation of the second subgroup has not been made previously, although Theiler & Downs (1973) set out a subgroup of eight viruses on the biological basis that no vector was known: their list includes Apoi, Modoc, Rio Bravo, Entebbe bat, Dakar bat, Bukalasa bat, Montana ML, and Cowbone Ridge viruses. Our tests show three cross-reactions linking the tick-borne subgroup (subgroup 1) with the unknown vector subgroup (subgroup 2), through Apoi virus and Negishi and Langat antisera.

The 26 mosquito-borne flaviviruses in our study differ from the 26 viruses listed by Theiler & Downs (1973) by their inclusion of Potiskum virus, which was not available to us, and by their omission of Chuku virus, which they identify as a strain of Spondweni. They point out that the four Dengue types form one complex of closely related viruses, they place West Nile, Japanese B, St Louis and Murray Valley encephalitis viruses in a second complex, with yellow fever, Uganda S, Wesselsbron and Banzi viruses in a third.

Our study recognizes somewhat different relationships within the mosquito-borne viruses. We confirm the close relationship between West Nile, Japanese B, St Louis and Murray Valley encephalitis viruses, and our subgroup 3 also brings in Usutu, an African virus, and the four Australian viruses, Kunjin, Kokobera, Stratford and Alfuy.

There are five cross-relationships between subgroups 2 and 3, through Entebbe bat virus reacting with Japanese B and West Nile antisera, Bukalasa bat virus with Japanese B antiserum, and Rio Bravo virus with Japanese B and Murray Valley encephalitis antisera.

Subgroup 4 contains three African viruses. Chuku virus was first described as a strain of Zika virus (Macnamara, 1954) and later as a strain of Spondweni virus (Clarke, reported in Theiler & Downs, 1973). Our studies indicate that Spondweni, Zika and Chuku viruses, although closely related, are clearly distinguishable. Spondweni antiserum neutralizes St Louis virus in subgroup 3.

Subgroup 5 brings together for the first time a virus isolated in Israel from turkeys with meningoencephalitis, Ntaya virus isolated from mosquitoes in Africa, and Tembusu virus isolated from mosquitoes collected in Malaysia. It is of interest that human sera collected in Malaysia have been found to neutralize Ntaya virus.

Our subgroup 6 confirms the close association between Banzi and Uganda S viruses, and

their relationship to an Australian virus, Edge Hill. Our findings do not place yellow fever and Wesselsbron viruses in this subgroup.

Subgroup 7 contains the 4 Dengue type viruses, but our findings do not confirm that the closest relationships are between types 1 and 3, and types 2 and 4.

We wish to thank Mr G. A. Davies for technical assistance.

REFERENCES

CASALS, J. (1957). The arthropod-borne group of animal viruses. *Transactions of the New York Academy of Sciences, Series* 2 **19**, 219–235.

LEIBOVITZ, A. (1963). The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *American Journal of Hygiene* **78**, 173–180.

MACNAMARA, F. N. (1954). Zika virus: a report on three cases of human infection during an epidemic of jaundice in Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **48**, 139–145.

MADRID, DE, A. T. & PORTERFIELD, J. S. (1969). A simple micro-culture method for the study of group B arboviruses. *Bulletin of the World Health Organization* **40**, 113–121.

MADRID, DE, A. T. (1972). Studies of flaviviruses (Group B arboviruses) with particular reference to antigenic composition. Thesis submitted to the Council for National Academic Awards for the degree of Doctor of Philosophy.

THEILER, M. & DOWNS, W. G. (1973). In *The arthropod-borne viruses of vertebrates*. New Haven and London: Yale University Press.

(*Received* 15 *October* 1973)