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ANTIGENIC RELATEDNESS OF SELECTED FLAVIVIRUSES: STUDY WITH HOMOLOGOUS AND HETEROLOGOUS IMMUNE MOUSE ASCITIC FLUIDS

S.S. BABA(1), A.H. FAGBAMI(2) & O.D. OLALEYE (2)

SUMMARY

The antigenic relationship of 9 flaviviruses, Yellow fever (YF) , Wesselsbron (WSL) , Uganda S (UGS) , Potiskum (POT), West Nile (WN) , Banzi (BAN) , Zika (ZK) , Dengue type 1 (DEN-1) and Dengue type 2 (DEN-2), was assessed by cross-haemagglutination-inhibition (Cross-HI) and cross-complement fixation (Cross-CF) reactions between each of the viruses and their homologous immune mouse ascitic fluids. Titre ratios were calculated using the heterologous and homologous titres.

Cross-CF reactions revealed wider antigenic variations among viruses than Cross-HI reactions. There was no significant antigenic variation between WSL, POT and YF viruses using either of those methods. However, definite differences in antigenicity were observed between them and UGS, BAN and ZK viruses. There were no significant differences between UGS, BAN and ZK or between DEN-1 and DEN-2. The serological relationship among flaviviruses is important in establishing diagnosis and epidemiology of these infections in Africa.

KEYWORDS: Flaviviruses; Antigenic relationship; Cross-serological reactions; Africa.

INTRODUCTION

The genus flavivirus, contains some 70 closely related human and veterinary pathogens causing many serious illnesses. Most members of the group are arthropod-borne, and are separated into eight antigenic complexes based on cross-neutralization with polyclonal hyperimmune ascitic fluids against each member of the group, while some members have not been assigned to any of the complexes (MADRID & PORTERFIELD[19]: CALISHER et al.[9]; FRANCKI et al.[16]). The immunological cross-relationships existing among flaviviruses have been well documented (MONATH & NYSTROM[21]; OLALEYE et al.[22]; BABA[2,3]; BABA et al.[4,5,6,7,8]). Despite vigorous attempts to resolve the antigenic relatedness of this group of viruses, inconsistent and unexpected results are usually obtained even with monoclonal antibodies. Extensive serologic cross-reactivity in complement fixation (CF), immunofluorescence (IF), haemagglutination-inhibition (HI) and neutralization (N) tests are usually encountered when working with members of the group. It has been observed that the titres obtained when hyperimmune and immune sera reacted with heterologous antigens were often as high as and at times even higher than the titres observed with homologous antigen (HENDERSON et al.[17]; THEILER & DOWNS[24]; MADRID & PORTERFIELD[19]; WHO[28]; CALISHER et al.[9]; BABA[2,3]; BABA et al.[4,5,7]).

MACNAMARA et al.[18] suggested that prior exposure to some flaviviruses particularly Zika and Uganda S might modify the course of Yellow fever virus infections. Such serological evidence suggests the possibility of immunological interrelations among flaviviruses which could be one of the determinants in the observed pattern of scattered yellow fever immune sera in most parts of Africa (THEILER & DOWNS[24]). Serological cross-reactivity among viruses has been associated with sharing of closely related antigenic sites or epitopes and cross-reactivity in neutralization tests depends on epitopes at the surface of the virion on the envelope glycoprotein (TRENT[27]). It has been independently concluded by many workers that the flaviviruses cross-reactivity is important in establishing diagnosis and epidemiology of these infections in Africa.

In order to increase our understanding of the antigenic relatedness of flaviviruses, we have used the results of Cross-complement fixation (Cross-CF) and Cross-haemagglutination-inhibition (Cross-HI) reactions to determine the degree of antigenic relationship of 9 flaviviruses: Yellow fever Uganda S (UGS) , Wesselsbron (WSL) , Potiskum (POT) , West Nile (WN), Banzi (BAN), Zika (ZK), Dengue type 1 (DEN-1) and Dengue type 2 (DEN-2). Four of these viruses, YF, WSL, POT and ZK have not been assigned to any of the existing serocomplexes. The degree of antigenic relatedness is determined by the titre ratios, computed from the heterologous and homologous antibody titres. Considerable close relationships and differences were observed among members of the group.

(1) Department of Veterinary Microbiology & Parasitology, Faculty of Veterinary Medicine, University of Maiduguri, Maiduguri, NIGERIA.
(2) Department of Virology, College of Medicine, University of Ibadan, Ibadan, NIGERIA.
Correspondence to: Dr. S.S. Baba, Department of Veterinary Microbiology & Parasitology, Faculty of Veterinary Medicine, University of Maiduguri, P.M.B. 1069, Maiduguri, NIGERIA.

TABLE 1
Viruses used in serological reactions

VIRUS	VIRUS NUMBER	PASSAGE HISTORY*	YEAR OF ISOLATION	LOCATION OF ISOLATION	SOURCE
Yellow fever	Hl14978	10th	1927	Ghana	Man
Wesselsbron	Ib-AN31956	8th	1968	Kano, Nigeria	Camel *(Camelus dromedarius)*
Uganda S	Ib-AN8829	7th	1966	Ife, Nigeria	Sentinel Mouse 615
Potiskum	Ib-AN10069	15th	1966	Fika, Nigeria	Giant Pouched Rat *(Cricetomys gambianus)*
West Nile	Ib-AN7019	8th	1965	Ibadan, NigeriaBird	*(Dicrurus adsimilis)*
Banzi	SA H336	Unknown	1956	South Africa	Man
Zika	Ib-H28444	Unknown	1968	Ibadan, Nigeria	Man
Denque type 1	Ib-H28328	Unknown	1968	Ibadan, Nigeria	Man
Dengue type 2	Ib-Hll234	Unknown	1966	Ibadan, Nigeria	Man

*Passage in suckling mouse brain

MATERIALS AND METHODS

Virus antigens

The viruses used in Cross-CF and Cross-HI reactions included: YF, UGS, WSL, POT, WN, BAN, ZK, DEN-1 and DEN-2 (Table 1). The virus antigens were prepared by sonication and sucrose-acetone extraction of infected suckling mouse brains as previously described by CLARKE & CASALS[11].

Homologous immune mouse ascitic fluid

The homologous antibody was prepared as immune mouse ascitic fluid (IMAF) against each of the flaviviruses used in the tests. They were prepared using the method described by TIKASINGH et al.[25]. Briefly, IMAF were raised with four intraperitoneal injections of the mixture of live virus and adjuvant given at weekly intervals. 0.2ml of sarcoma 180/TG cells was given 3 days after the third injection. Ascitic fluid was tapped by paracentesis from the distended abdomen of each mouse using sterile syringe and needle. The fluids were centrifuged at 349 g and supernatant stored in a mechanical freezer (-20°C) until used.

Cross-haemagglutination inhibition test

Cross-HI test was performed on each of the kaolin treated homologous IMAF according to the method of CLARKE & CASALS[11] adapted to microplate. Serial dilutions (starting dilution 1:10) of each IMAF were tested against all the virus antigens used in the test in a cross-HI test. Four to eight haemagglutination units of the antigen were used in the test.

Cross complement fixation test

The Cross-CF test was performed essentially using the method described by SEVER[23]. The IMAF was inactivated at 56°C for 30 min and tested in a serial two fold dilutions (starting dilution 1:4) against predetermined optimum dilutions of all antigens used in the test. The reciprocal of IMAF dilution given at least 3+ fixation was taken as the end point.

Determination of antigenic relatedness of flaviviruses

The antigenic relatedness of flaviviruses used in the study was determined from the results of Cross-HI and Cross-CF reactions. The degree of relatedness was computed from the titre ratios using the formula of ARCHETTI & HORSFALL[1] which states that $R = \sqrt{r1 \times r2}$. The ratio reflects the extent of antigenic differences between two viruses when both viruses and their homologous IMAFs were used in reciprocal cross-serological reactions. The ratio r1 is determined by dividing the heterologous titres obtained with flavivirus 2 by the homologous titre obtained with flavivirus 1, and the ratio r2 is determined by dividing the heterologous titre obtained with flavivirus 1 by the homologous titre obtained with flavivirus 2.

$$\text{i.e. } r1 = \frac{\text{heterologous titre (flavivirus 2)}}{\text{homologous titre (flavivirus 1)}}$$

$$r2 = \frac{\text{heterologous titre (flavivirus 1)}}{\text{homologous titre (flavivirus 2)}}$$

The formula yields an antibody ratio (R) that expresses the antigenic relatedness between two viruses when both antigens and both IMAFs are used in cross-serological reactions. R values were

TABLE 2

Results of cross-haemagglutination inhibition tests with flaviviruses and their titre ratios using homologous immune mouse ascitic fluid.

VIRUS	HAEMAGGLUTINATION			INHIBITING ANTIBODY			TITRE IN IMAF		
	BAN	DEN-1	DEN-2	YF	POT	UGS	WN	WSL	ZK
BAN	1280	20	40	320	160	1280	160	160	640
DEN-1	-	1280	640	160	160	-	80	80	80
DEN-2	-	1280	1280	20	160	-	160	80	80
YF	160	320	160	1280	1280	160	320	1280	40
POT	160	160	80	640	1280	160	80	640	80
UGS	1280	40	40	160	320	1280	40	320	640
WN	80	160	160	320	320	80	1280	640	640
WSL	160	80	80	640	640	160	320	1280	40
ZK	640	80	80	40	160	320	160	80	1280
				Titre	Ratio*				
BAN	1.0	0.02	0.03	0.25	0.13	1.0	0.13	0.13	0.5
DEN-1	0	1.0	0.5	0.13	0.13	0	0.06	0.06	0.6
DEN-2	0	1.0	1.0	0.02	0.13	0	0.13	0.06	0.6
YF	0.13	0.25	0.13	1.0	1.0	0.13	0.25	1.0	0.03
POT	0.13	0.13	*0.06*	0.5	1.0	0.13	0.06	0.5	0.06
UGS	1.0	0.03	0.03	0.03	0.25	1.0	0.03	0.25	0.5
WN	0.06	0.13	0.13	0.25	0.25	0.06	1.0	0.5	0.5
WSL	0.13	0.06	0.06	0.5	0.5	0.13	0.25	1.0	0.03
ZK	0.5	0.06	0.06	0.03	0.13	0.25	0.13	0.06	1.0

*Titre ratio: heterologous titre divided by homologous titre
-. Negative.

calculated from a geometric mean antibody titre obtained from a minimum of three tests. A homologous R value is by definition 1 and R value of 1 or ≥ 0.5 indicate antigenic similarity between two flaviviruses. Significant differences were determined based on Students' two-tailed t-test.

RESULTS

Table 2 shows the results of end point titrations for Cross-HI reactions using homologous IMAFs against flavivirus antigens used in the tests, and their titer ratios computed from geometric mean titers of homologous and heterologous antibodies. No significant difference ($P>0.05$) between WSL, YF and POT viruses and between BAN and UGS as well as between DEN-1 and DEN-2. Significant differences were however, observed between them and other viruses used in Cross-HI reactions. By means of ratio $R = \sqrt{r1 \times r2}$ previously described, the extent of cross relationship between two viruses, as indicated by two heterologous titer ratios, can be expressed in a single figure (Table 3). With YF and POT, R= 0.7; YF and WSL. R= 0.7: UGS and BAN. R = 1.0: and DEN-1 and DEN-2. R =1.0 indicating no significant antigenic differences between each pair. However marked differences were observed for example, between WSL and

TABLE 3
Antigenic relatedness of flaviviruses expressed as R - value calculated from HI titre ratios

VIRUS	R Value $\sqrt{r\ 1} \times 2$								
	BAN	DEN-1	DEN-2	YF	POT	UGS	WN	WSL	ZK
BAN	1.0	0	0	0.18	0.13	1.0	0.09	0.13	0.5
DEN-1		1.0	0.7	0.18	0.13	0	0.09	0.06	0.06
DEN-2			1.0	0.13	0.09	0	0.13	0.06	0.06
YF				1.0	0.7	0.06	0.25	0.7	0.03
POT					1.0	0.18	0.12	0.5	0.09
UGS						1.0	0.04	0.18	0.35
WN							1.0	0.35	0.25
WSL								1.0	0.04
ZK									1.0

R value $\geq$ 0.5 indicates antigenic similarity.

ZK, R = 0.04 (Table 3). The observed pattern of Cross-CF reactions was slightly different from that of Cross-HI tests based on differences in R values (Tables 4 and 5) . Cross-CF reactions revealed wider antigenic variations among viruses than Cross-HI reactions. Generally, there were no significant antigenic variations between WSL, POT, and YF using either of the two reactions. However, definite differences in antigenicity were observed between them and UGS, BAN and ZK viruses. In addition, antigenic similarities were also noted between DEN-1 and DEN-2 and between UGS, BAN and ZK.

DISCUSSION

The diagnosis and understanding of the epidemiology of flavivirus infections in endemic areas of Africa is continuously hampered mainly by extensive serological cross-relationships among members of the group. It has been observed that the titers obtained when hyperimmune and immune sera reacted with heterologous antigens were often as high as and at times even higher than the titers observed with homologous antigen (HENDERSON et al.[17]; THEILER & DOWNS[24]; MADRID & PORTERFIELD[19]; WHO[28]; BABA et al.[4,5,6,7]; CALISHER et al.[9]; BABA[2,3]). Serological diagnosis is usually resorted to when virus isolation attempts fail or where there is need for extensive survey involving a large population. However, in places where more than one flavivirus occur, interpretation of serological tests is usually difficult because of the immunological cross reactions that occur between them. EVANS et al.[13] reported a nationwide serum survey of Argentinean military recruits between 1965 and 1966. They observed that a number of sera had HI antibodies to more than one flavivirus. In a related study during the 1970 yellow fever epidemic in Okwoga district, Benue Plateau State of Nigeria, MONATH et al.[20] examined the serological responses in persons with or without pre-existing heterologous group flavivirus immunity. Various patterns of serological reactions were obtained in HI, CF, and N tests which necessitated the design of criteria for the interpretation of experimental results. The difficulties in interpretation of results of serological reactions using classical serological methods e.g. CF, HI and N had resulted in the development of more sensitive and specific techniques. For instance, MONATH & NYSTROM[21] developed enzyme linked immunosorbent assay (ELISA) for detection of YF IgM in serum and antigen from clinical specimen. The method has been accepted generally as being sensitive and specific and can be used for diagnosis of flavivirus infections especially in endemic environments. Recently, BABA et al.[8] described the development of a relatively inexpensive procedure for the specific detection of WSL virus IgM in human sera using flavivirus HI test adapted to solid-phase immunosorbent technique (SPIT). In spite of these rigorous attempts to resolve the problem of antigenic relatedness of flaviviruses, inconsistent and unexpected results are usually obtained even with monoclonal antibodies.

In this study, attempts have been made to establish the degree of antigenic relatedness among members of the group. Some members (WSL, POT and YF) were found to be closely related while considerable differences were observed between them and UGS, BAN and ZK viruses. In addition, there was no difference between UGS, BAN and ZK or between DEN-1 and DEN-2. Although the role of pre-infection flavivirus antibodies in the modification of the outcome of any flavivirus infection has not been well defined, we could speculate from the results of this study that preexisting flavivirus antibodies for instance, to WSL and POT viruses could modify the course of YF virus infections. HENDERSON et al.[17] have demonstrated that monkeys infected with WSL virus were resistant to a subsequent challenge with virulent YF virus and indeed,

TABLE 4
Results of cross-complement fixation tests with flaviviruses and their titre ratios using homologous immune mouse ascitic fluids

VIRUS	COMPLEMENT FIXING ANTIBODY TITRE IN IMAF								
	BAN	**DEN-1**	**DEN-2**	**YF**	**POT**	**UGS**	**WN**	**WSL**	**ZK**
BAN	64	2	-	2	-	32	4	4	32
DEN-1	4	16	16	2	2	2	4	2	4
DEN-2	4	16	32	2	2	4	4	2	2
YF	-	-	-	64	32	2	16	32	-
POT	4	-	-	32	32	4	4	16	4
UGS	16	-	-	-	8	32	-	-	8
WN	-	-	-	2	-	-	64	4	8
WSL	2	2	2	64	32	2	4	64	2
ZK	16	-	-	32	-	16	*16*	-	64
					Titre Ratio*				
BAN	1.0	0.03	0	0.03	0	0.5	0.06	0.06	0.5
DEN-1	0.25	1.0	1.0	*0.13*	0.13	0.13	0.25	0.13	0.25
DEN-2	0.13	0.5	1.0	0.06	0.06	0.06	0.13	0.06	0.06
YF	0	0	0	1.0	0.5	0.03	0.25	0.5	0
POT	0.13	0	0	1.0	1.0	0.13	0.13	0.5	0.13
UGS	0.5	0	0	0	0.25	1.0	0	0	0.25
WN	0	0	0	0.03	0	0	1.0	0.06	0.13
WSL	0.03	0.03	0.03	1.0	0.5	0.03	0.06	1.0	0.03
ZK	0.25	0	0	0.5	0	0.25	0.25	0	1.0

*Titre ratio = heterologous titre divided by homologous titre
- Negative

circulated no detectable amount of YF virus following challenge. Based on the results of serological surveys among human and animal populations in Nigeria, it has been suggested that prior exposure to some Group B viruses (flaviviruses) might modify the course of flavivirus infections (MONATH et al.[20]; THEILER & DOWNS[24]; FAGBAMI & OJEH[14]; BABA et al.[5]; CALISHER et al.[9]; TOMORI et al.[26]). Two types of immune responses can be observed in flavivirus infections, following primary contact with a virus, homologous HI and CF antibodies appear but antibodies to other flaviviruses are absent or of low titer. On the other hand, in the case of a secondary infection due to another flavivirus, heterologous antibodies appear at a high level, often greater than homologous titers; this is particularly so when the flavivirus contacts follow in close succession (DIGOUTTE et al.[12]) . Flavivirus neutralizing antibodies are of later appearance and last for many years (probably for life) . However, serum neutralization tests are more delicate and more costly than HI and CF tests and difficult to employ in extensive serological surveys in endemic environment where many flaviviruses circulate. Therefore, in flavivirus endemic zones, HI and CF tests are widely used in serological surveys to obtain estimates of the prevalence of flavivirus antibodies in a determined location. In such situations, sera have to be tested against antigens prepared from related flaviviruses in the zone. This study has provided information that could be used as a model for designing and interpreting serological reactions using panels of flavivirus antigens. Antibodies to the 9 flaviviruses analyzed in this study have been detected in sera of man

TABLE 5
Antigenic relatedness of flaviviruses expressed as R-value calculated from CF titre ratio

VIRUSES	R Value $\sqrt{r1 \times r2}$								
	BAN	**DEN-1**	**DEN-2**	**YF**	**POT**	**UGS**	**WN**	**WSL**	**ZK**
BAN	1.0	0.08	0	0	0	0.5	0	0.04	0.35
DEN-1		1.0	0.7	0	0	0	0	0.06	0
DEN-2			1.0	0	0	0	0	0.04	0
YF				1.0	0.7	0	0.08	0.7	0
POT					1.0	0.18	0	0.5	0
UGS						1.0	0	0	0.25
WN							1.0	0.06	0.18
WSL								1.0	0
ZK									1.0

R Value $\geq$ 0.5 indicates antigenic similarity.

and animals in Nigeria and isolation of many of them have been made from man, animals and arthropod in the country. POT virus has only been isolated from liver obtained from *Cricetomys gambianus* (giant pouched rat) at Fika in Nigeria (CAUSEY et al.[10], FAGBAMI et al.[15]). Human and domestic animal isolates of the virus have not been obtained, but antibodies have been detected in human and animal sera (FAGBAMI et al.[15]). The virus has been found to be closely related to several other flaviviruses based on the results of cross serological reactions with other flaviviruses (CALISHER et al.[9], FAGBAMI et al.[15], THEILER & DOWNS[24]). Most members of the genus flavivirus have been separated into eight antigenic complexes, while four of them YF, WSL, POT, ZK have not been assigned to any of the existing serocomplexes. The analysis of flavivirus antigenic relatedness carried out in this study may not completely demonstrate the degree of relationships among the viruses. Nevertheless, it could assist in planning and interpretation of serological reactions on human and animal sera in this environment. The final determination of the genetic relatedness of flaviviruses would require the availability of information on their nucleotide sequences.

RESUMO

Relação antigênica de determinados Flavivirus: estudo com fluidos ascíticos homólogos e heterólogos de camundongos imunes

A relação antigênica de 9 Flavivirus, Febre amarela (YF), Wesselsbron (WSL), Uganda S (UGS), Potiskum (POT), West Nile (WN), Banzi (BAN), Zika (ZK), Dengue tipo 1 (DEN-1) e Dengue tipo2 (DEN-2), foi avaliada por reação de inibição da hemaglutinação cruzada (cross-HI) e reação de fixação do complemento cruzada (Cross-CF) entre cada um dos virus e seu fluido ascítico homólogo em camundongos. Médias de títulos foram calculadas usando os títulos heterólogos e homólogos.

Reações cruzadas CF revelaram maiores variações antigênicas entre virus do que reações cruzadas HI. Não houve variação antigênica significativa entre virus WSL, POT e YF usando cada um dos métodos. Todavia, diferenças definidas da antigenicidade foram observadas entre eles e os vírus UGS, BAN e ZK. Não existiram diferenças significativas entre UGS, BAN e ZK ou entre DEN-1 e DEN-2. A relação sorológica entre Flavivirus é importante para se estabelecer o diagnóstico e a epidemiologia destas infecções na África.

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