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USE OF HYPERIMMUNE MOUSE ASCITIC FLUIDS FOR ARBOVIRUS DIFFERENTIATION BY INDIRECT IMMUNOFLUORESCENCE AND CONVENTIONAL SEROLOGY

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Summary. — The cross-reactivity of 22 arbovirus species (alphaviruses; tick- and mosquito-borne flaviviruses; and selected bunyaviruses) was tested with monovalent immune mouse ascitic fluids by indirect immunofluorescence (IIF) in comparison with classical serological reactions (virus neutralization — VN; haemagglutination inhibition — HI; and complement fixation — CF — reactions). Known relationships within the virus groups studied were confirmed. As to the differentiation limits, the VN test was followed by IIF. Evaluation of the ratio of heterologous to homologous antibody activities showed that with the exception of antigenically closely related arboviruses (Western equine encephalomyelitis — Sindbis; Japanese encephalitis — Murray Valley encephalitis; dengue viruses; California — Ťahyňa), most arboviruses within the antigenic complexes tested could be relatively reliably differentiated by IIF.

Key words: arboviruses; *differentiation*; *immune ascitic fluids*; *immunofluorescence*; *serology*

Introduction

Antigenic relationships studied and demonstrated in particular by Casals and his group (Casals, 1957, 1963, 1971; etc.) and a number of others by so-called classical serological methods (virus neutralization — VN; haemagglutination inhibition — HI; and complement fixation — CF — tests) and gel immunodiffusion served as a basis of previous arbovirus classification. Conventional serology along with immunoprecipitation methods, especially with the use of absorption or chromatographic techniques, represents a comparatively sensitive biological approach which so far led to the differentiation of over 50 antigenic groups and complexes of arboviruses (Berge, 1975; Karabatsos, 1978). Even after the introduction of modern taxonomy based on morphological and molecular biological properties of virions and their RNA, immunological relationships remain a basic criterion in distinguishing species within genera of viruses and serve as a basis in laboratory differential diagnosis of clinically and epidemiologically frequently similar

arbovirus infections. From this point of view only a few scattered informations are available on the specificity and impact for differential diagnosis of immunofluorescence techniques, especially on the basis of ascitic antibody.

In recent years we have prepared a set of 22 highly active hyperimmune mouse ascitic fluids (IAF) against arboviruses causing important human diseases. We used them to study the differentiating ability of indirect immunofluorescence (IIF) in comparison with classical serological reactions. In view of the incomplete set of viruses and of the fact that non-absorbed IAF were used, the results presented below should not be considered as an attempt at a detailed antigenic analysis of the viruses examined.

Materials and Methods

Viruses. In our comparative study we used 22 arboviruses belonging to 4 genera in the families *Togaviridae* and *Bunyaviridae*. Genus *Alphavirus*: Eastern (EEE), Western (WEE) and Venezuelan (VEE) equine encephalomyelitis, Chikungunya (CHIK) and Sindbis (SIN) viruses; genus *Flavivirus*: Central European tick-borne encephalitis (TBE) virus strain Hypr, Omsk haemorrhagic fever (OHF) and Kyasanur Forest disease (KFD) viruses of the TBE complex; mosquito-borne Japanese encephalitis (JE), Murray Valley encephalitis (MVE), St. Louis encephalitis (SLE) and West Nile (WN) viruses; dengue (D-1, 2, 3, 4) viruses; and yellow fever (YF) viruses. *Bunyaviridae*: Bunyamwera (BUN), California (CAL) and Ťahyňa (TAH) viruses of the genus *Bunyavirus* and two types of phlebotomus fever virus — Neapolitan (SFN) and Sicilian (SFS). For inoculation of cell cultures, the viruses were used in the form of suckling mouse brain suspensions in borate buffer pH 9.0 with 1% bovine albumin (BA) and antibiotics.

Antiviral antibodies were prepared by two-phase immunization of mice with homologous virus suspensions with subsequent ascites induction by sarcoma 180 cells. For details see Hronovský *et al.* (1972) and Hronovský (1978).

Virus neutralization (VN) tests were done in tube cultures of CV-1 monkey kidney cells (for their properties and mode of cultivation see Hronovský *et al.*, 1978). Serial twofold dilutions of IAF (starting with 1 : 4) were mixed with 80—250 TCD_{50} of virus and incubated for 90 min at 37 °C before inoculation.

Complement fixation (CF; box titration) and haemagglutination inhibition (HI) tests were done by standard microtechniques (Hammon and Sather, 1974) on plastic panels with sucrose-acetone (SA) antigens (Clarke and Casals, 1958) inactivated, if necessary, by 0.1% beta-propiolactone. Before used in HI tests, individual antigens were examined for optimization of haemagglutination conditions (temperature, pH, BA contents in diluent buffer). HI titres were read against 4 haemagglutinating units of antigen.

Indirect immunofluorescence (IIF). Activity of the IAF was assayed in coverslip cultures of CV-1 cells infected with the respective viruses and fixed at appropriate intervals after inoculation (p.i.). The antigens were visualized by commercial anti-mouse fluorescein isothiocyanate-labelled conjugate in a mixture with Evans blue. Before test, the optimal working dilutions of both components were determined. The IIF titres of most IAF against homologous antigens varied from 256 to 1024 and the working dilutions varied from 1 : 32 to 1 : 128.

As a rule, lyophilized IAF were used. The specificity of the reactions was checked by appropriate controls.

Results

The results of cross VN, HI, CF and IIF tests on 5 alphaviruses, 3 tick-borne flaviviruses, 4 mosquito-borne flaviviruses of the JE subgroup, 5 mosquito-borne flaviviruses of the dengue-YF subgroup and 5 bunyaviruses are summarized in Tables 1—5. In view of the unequal starting antibody

Table 1. Cross-reactions among alphaviruses

Viral antigen		IAF to virus									
		VEE		EEE		WEE		CHIK		SIN	
		I	II	I	II	I	II	I	II	I	II
VEE	VNT	**1024**	—	8	1/128	8	1/25	< 6	< 1/75	< 6	< 1/30
	HIT	**2560**	—	20	1/64	40	1/32	10	1/128	20	1/64
	CFR	**1024**	—	8	1/64	16	1/64	< 4	< 1/64	4	1/256
	IIF	**1024**	—	32	1/16	32	1/16	8	1/32	4	1/64
EEE	VNT	< 6	< 1/170	**1024**	—	< 6	< 1/33	6	1/75	< 6	< 1/30
	HIT	20	1/128	**1280**	—	160	1/8	20	1/64	20	1/64
	CFR	16	1/64	**512**	—	64	1/16	< 4	< 1/64	16	1/64
	IIF	64	1/16	**512**	—	64	1/8	8	1/32	4	1/64
WEE	VNT	< 6	< 1/170	< 6	< 1/170	**200**	—	< 6	< 1/175	< 6	< 1/30
	HIT	80	1/32	40	1/32	**1280**	—	20	1/64	320	1/4
	CFR	16	1/64	16	1/32	**1024**	—	< 4	< 1/64	128	1/8
	IIF	32	1/32	32	1/16	**1024**	—	8	1/32	128	1/4
CHIK	VNT	< 6	< 1/170	< 6	< 1/170	< 6	< 1/33	**450**	—	< 6	< 1/32
	HIT	40	1/64	20	1/64	40	1/32	**1280**	—	20	1/64
	CFR	16	1/64	8	1/64	8	1/128	**256**	—	32	1/32
	IIF	8	1/128	8	1/64	16	1/32	**256**	—	4	1/64
SIN	VNT	< 6	< 1/170	< 6	< 1/170	< 6	< 1/33	< 6	< 1/75	**180**	—
	HIT	40	1/64	10	1/128	320	1/4	10	1/128	**1280**	—
	CFR	4	1/256	4	1/128	256	1/4	< < 4	< 1/64	**1024**	—
	IIF	32	1/32	16	1/32	128	1/4	16	1/16	**256**	

I — Titre determined in the given test.
II — Ratio of titre with heterologous and homologous antigen.
In the VNT, the virus doses (TCD_{50}) used were: EEE and CHIK — 100; VEE — 150; WEE — 250; and SIN — 160.

Table 2. Cross reactions among tick-borne flaviviruses

Viral antigen		IAF to virus					
		TBE		OHF		KFD	
		I	II	I	II	I	II
TBE	VNT	**1024**	—	64	1/5.5	28	1/5.7
	HIT	**640**	—	80	1/8	320	1/2
	CFR	**256**	—	64	1/4	128	1/8
	IIF	**512**	—	64	1/8	128	1/8
OHF	VNT	64	1/16	**350**	—	45	1/3.6
	HIT	160	1/4	**640**	—	320	1/2
	CFR	128	1/2	**256**	—	64	1/8
	IIF	64	1/8	**512**	—	16	1/64
KFD	VNT	< 10	< 1/100	< 10	< 1/35	**160**	—
	HIT	160	1/4	80	1/8	**640**	—
	CFR	64	1/4	16	1/16	**512**	—
	IIF	16	1/32	32	1/16	**1024**	—

I and II as in Table 1.
In the VNT, the virus doses (TCD_{50}) used were: TBE — 160; and OHF and KFD — 100.

levels of the individual IAF, the degree of cross-reactions was also expressed, for sake of better comparison, by the mutual ratio of heterologous to homologous antibodies.

The experiments confirmed the known antigenic relationships between certain viruses within the tested groups of the family *Togaviridae* manifested by one- or two-sided higher degree of cross-reactions. In alphaviruses, in addition to relationships between equine encephalomyelitis viruses, HI, CF and IIF tests confirmed the close two-sided relationship of WEE with the prototype SIN alphavirus (Casals, 1963; Mussgay, 1967). In flaviviruses we also confirmed the relationships between the tested representatives of the TBE complex (especially TBE — OHF), between 4 mosquito-borne flaviviruses of the JE subgroup (especially JE — MVE) and between the individual dengue serotypes (De Madrid and Porterfield, 1974; Berge, 1975; etc.). IAF to YF, tested with dengue viruses was, by contrast, highly specific and gave a marked reaction in HI, CF and IIF tests only with D-4 virus. As expected, in the arbovirus groups mentioned the VN tests unambiguously showed the highest differentiating ability and, with the exception of some alphaviruses, the highest degree of cross-reactivity was shown by HI antibodies. IAF against both groups of mosquito-borne flaviviruses were cross-tested also with antigens of the second group. Without exception, VN tests proved to be unequivocally specific; weak antigenic relationships (e. g. YF, vs. WN, D-4 vs. SLE) were observed in a part of the HI, CF and IIF reactions, mainly at the level of the starting antibody dilutions.

Table 3. Cross reactions among mosquito-borne flaviviruses (JE subgroup)

Viral antigen		IAF to virus							
		JE		MVE		SLE		WN	
		I	II	I	II	I	II	I	II
JE	VNT	**708**	—	10	1/18	< 6	< 1/105	6	1/30
	HIT	**1280**	—	320	1/2	640	1/4	80	1/16
	CFR	**512**	—	128	1/8	32	1/32	32	1/8
	IIF	**2048**	—	256	1/4	128	1/16	64	1/8
MVE	VNT	45	1/16	**180**	—	14	1/43	10	1/18
	HIT	320	1/4	**640**	—	320	1/8	80	1/16
	CFR	128	1/4	**1024**	—	32	1/32	32	1/8
	IIF	128	1/16	**1024**	—	32	1/64	16	1/32
SLE	VNT	< 6	< 1/120	8	1/22	**630**	—	< 6	< 1/30
	HIT	640	1/2	320	1/2	**2560**	—	160	1/8
	CFR	64	1/8	64	1/16	**1024**	—	32	1/8
	IIF	64	1/32	32	1/32	**2048**	—	32	1/16
WN	VNT	< 6	< 1/120	10	1/18	< 6	< 1/105	**180**	—
	HIT	640	1/2	160	1/4	320	1/8	**1280**	—
	CFR	64	1/8	64	1/16	64	1/16	**256**	—
	IIF	64	1/32	32	1/32	32	1/64	**512**	—

I and II as in Table 1.
In the VNT, the virus doses (TCD_{50}) used were: JE — 100; MVE — 250; SLE — 200; and WN — 160.

Cross-reactions with 5 members of the antigenically heterogeneous family *Bunyaviridae* confirmed the close relationship of both representatives of the California subgroup of the genus *Bunyavirus* CAL and TAH (Casals, 1962; Lindsey *et al.*, 1976). The other heterologous VN and IIF tests were negative. In HI and especially CF tests, distant antigenic relationships between some viruses (TAH vs. BUN, SFN, SFS; BUN vs. SFN; SFN vs. SFS) were observed, mainly at the level of starting antibody dilutions only.

As concerns differentiation by immunofluorescence, with the use of IAF with a high antibody activity the IIF was mostly significantly more specific than the HI test. IIF was comparable to, and in many instances (e. g. with viruses of the TBE complex and most of the cross-reacting mosquito-borne flaviviruses) its resolving power was higher than that of the CF reaction. The intensity of specific fluorescence in heterologous reactions, with the exception of the lowest dilutions tested (1 : 4—1 : 8) was markedly lower than in homologous reactions of the same antibody.

Discussion

The cross-reactions within (or also outside) serological groups of arboviruses are in principle due to common antigenic determinants (amino acid sequences

Table 4. Cross-reactions among mosquito-borne flaviviruses (dengue subgroup)

Virus antigen		IAF to virus									
		D−1		D−2		D−3		D−4		YF	
		I	II	I	II	I	II	I	II	I	II
D−1	VNT	**80**	—	10	1/16	20	1/18	10	1/13	< 6	< 1/170
	HIT	**80**	—	40	1/8	160	1/8	160	1/32	10	1/64
	CFR	**128**	—	32	1/8	256	1/2	128	1/4	< 4	< 1/32
	IIF	**128**	—	64	1/2	32	1/8	32	1/32	< 4	< 1/128
D−2	VNT	6	1/13	**160**	—	25	1/14	10	1/13	< 6	< 1/170
	HIT	10	1/8	**320**	—	320	1/4	320	1/16	4	1/32
	CFR	32	1/4	**256**	—	128	1/4	128	1/4	< 4	< 1/32
	IIF	32	1/4	**128**	—	32	1/8	32	1/32	< 4	< 1/128
D−3	VNT	< 6	< 1/13	< 6	< 1/27	**354**	—	10	1/13	< 6	< 1/170
	HIT	10	1/8	80	1/4	**1280**	—	640	1/8	10	1/64
	CFR	16	1/8	32	1/8	**512**	—	128	1/4	< 4	< 1/32
	IIF	8	1/16	16	1/8	**512**	—	256	1/4	< 4	< 1/128
D−4	VNT	6	1/13	8	1/20	10	1/35	**128**	—	< 6	< 1/170
	HIT	20	1/4	160	1/2	640	1/2	**5120**	—	40	1/16
	CFR	16	1/8	64	1/4	256	1/2	**512**	—	8	1/16
	IIF	16	1/8	32	1/4	128	1/4	**1024**	—	16	1/32
YF	VNT	< 6	< 1/13	< 6	< 1/27	< 6	< 1/59	< 6	< 1/21	**1024**	—
	HIT	10	1/8	40	1/8	80	1/16	160	1/32	**640**	—
	CFR	4	1/32	< 4	< 1/64	< 4	< 1/128	< 4	< 1/128	**128**	—
	IIF	< 4	< 1/32	< 4	< 1/32	< 4	< 1/128	4	1/256	**512**	—

I and II as in Table 1.
In the VNT, the virus doses (TCD_{50}) used were: D−1, D−2 and YF — 100; D−3 — 150; and D−4 — 125.

Table 5. Cross-reactions with selected bunyaviruses

Virus antigen		IAF to virus									
		BUN		CAL		ATH		SFN		SFS	
		I	II	I	II	I	II	I	II	I	II
BUN	VNT	**2220**	—	< 6	< 1/470	< 6	< 1/340	< 6	< 1/85	< 6	< 1/116
	HIT	**1280**	—	< 10	—	10	—	<10	< 1/64	< 10	< 1/32
	CFR	**1024**	—	4	1/256	4	1/512	8	1/128	4	1/256
	IIF	**2048**	—	< 4	<1/256	< 4	< 1/512	< 4	< 1/256	< 4	< 1/256
CAL	VNT	6	1/370	**2820**	—	512	1/4	< 6	< 1/85	< 6	< 1/116
	HIT	NT	—	NT	—	NT	—	NT	—	NT	—
	CFR	8	1/128	**1024**	—	128	1/16	8	1/128	4	1/256
	IIF	< 4	< 1/512	**1024**	—	256	1/8	< 4	< 1/256	< 4	< 1/256
TAH	VNT	< 6	< 1/370	316	1/9	**2048**	—	6	< 1/85	< 6	< 1/116
	HIT	NT	—	NT	—	NT	—	NT	—	NT	—
	CFR	8	1/128	512	1/2	**2048**	—	8	1/128	4	1/256
	IIF	< 4	< 1/512	128	1/8	**2048**	—	< 4	1/256	< 4	< 1/256
SFN	VNT	< 6	< 1/370	< 6	< 1/470	< 6	<1/340	**512**	—	< 6	<1/116
	HIT	10	1/128	10	—	10	—	**640**	—	20	1/16
	CFR	8	1/128	4	1/256	4	1/512	**1024**	—	4	1/256
	IIF	< 4	< 1/512	< 4	< 1/256	< 4	<1 /512	**2048**	—	< 4	< 1/256
SFS	VNT	< 6	< 1/370	<6	< 1/470	6	< 1/340	6	< 1/85	**700**	—
	HIT	< 10	< 1/128	< 10	—	10	—	20	1/32	**320**	—
	CFR	8	1/128	4	1/256	4	1/512	8	1/128	**1024**	—
	IIF	< 4	< 1/512	< 4	< 1/256	< 4	< 1/512	< 4	< 1/256	**1024**	—

I and II as in Table 1.
NT — not tested.

of structural virion proteins) or eventually also common glycoproteins of host origin (as a consequence of virus replication in cells of the same species) which, after a natural or experimental infection induce antibodies giving heterologous reactions (Porterfield, 1975). Studies on mouse, rabbit and guinea pig sera showed that the relative degree of antigenic overlap within the groups varies with the tests used. The specificity of the latter is not the same in all groups so that a test suitable only for group identification in one group is suitable for specific identification in another group. For example alpha- and flaviviruses show the most antigenic cross-reactions in the HI test which represents a group reaction, less cross-reactions in the CF and least in the VN test. The latter test is used for type-specific identification of togaviruses. In general, our results are in accordance with these conclusions which have been later confirmed by experiments on mouse IAF (Tikasingh et al., 1966; Gaidamovich *et al.*, 1969; Deig, 1974; etc.).

The immunofluorescence technique, combining to a certain degree the advantages of microscopic visualization and immunological demonstration of antigens, was used by a number of authors in attempts at arbovirus differentiation (e. g., Kunz, 1964; Atchinson *et al.*, 1966; Buckley and Clarke, 1970; and others). The results obtained are hardly comparable because of the different techniques and experimental conditions used (direct, indirect or complement IF; the use of absorption techniques; combination with microphotometry; sera of different efficiency; different detection substrates). It may be concluded, however, that IF and especially IIF may be used for a reliable differentiation and identification of arboviruses at the level of basic antigenic groups that are clearly distinct by classical serology.

In agreement with others (Gajdamovič *et al.*, 1973; and others) who, in parallel with us, were interested in the use of IAF for immunofluorescent detection of arboviruses, we already previously reached similar conclusions on a few virus models. We confirmed that IIF reactions with hyperimmune ascitic fluids make it possible to reliably differentiate taxonomic genera and antigenic groups within genera (e. g., the complexes of TBE, JE and dengue within the *Flavivirus* genus) and that the degree of group differentiation by immunofluorescence gives the highest correlation with CF findings (Hronovský, 1978).

These findings were now extended by a series of cross IIF reactions on a much broader range of highly active IAF. The present data make it possible to conclude that differentiation by IIF is reliable if the homologous titres are at least by three dilution steps (8-fold) higher than the heterologous titres. This corresponds to the usual difference between staining titre and the working dilution of IAF in the homologous reaction. With the exception of viruses showing a close two- or one-sided antigenic relationship (in our collection especially alphaviruses WEE-SIN; mosquito-borne flaviviruses JE-MVE; dengue viruses, mainly D-1 — D-2 and D-3 — D-4; and bunyaviruses CAL-TAH), most of the other viruses within the groups tested could be identified and mutually differentiated with a high degree of reliability based on this criterion with the use of optimal IAF dilutions.

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