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THE USE OF SCANNING ELECTRON MICROSCOPY IN THE STUDY OF HAEMAGGLUTINATION INDUCED BY VARIOUS ARBOVIRUSES

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Summary. — Scanning electron microscopy was used to study haemagglutination by 10 arboviruses belonging to the Togaviridae and Bunyaviridae families. The pictures obtained did not allow to clarify the precise mechanism of haemagglutination by these viruses. The data suggest that no fundamental differences exist in the mechanism of haemagglutination between these two arbovirus families.

Key words: *arboviruses*; *haemagglutination*; *scanning electron microscopy*

Introduction

It is known that the characteristics of haemagglutinating antigens of arboviruses, obtained by classical extraction techniques of Clarke and Casals (1958), may show considerable variation. While most viruses of the Togaviridae family yield potent haemagglutinins, producing compact and firm agglutinates, other viruses (in particular, those of the Bunyaviridae family) have only weak haemagglutinins in low titre, leading to the formation of fragile agglutinates.

Many techniques have been put forward with the aim to improve haemagglutination (Saturno, 1967; Ardouin *et al.*, 1969; Beaty *et al.*, 1977; Traavik, 1977), but there have been no attempts to discover the reason for the differences which exist between different viruses. Tawara *et al.* (1976) used successfully scanning electron microscopy (SEM) to visualize precisely how the particles of influenza B virus become adsorbed to the surface of chicken red blood cells (RBC), producing intercellular bridging responsible for the Hirst phenomenon (Hirst, 1941, 1942). We, therefore, decided to use this technique with the aim to find out whether apparent differences between the haemagglutinins of the viruses of families Togaviridae and Bunyaviridae would be reflected by any fundamental ultrastructural differences.

Materials and Methods

Virus strains. Table 1 summarizes the 10 arboviruses used. Six of them belong to the family Togaviridae. Two of these are alphaviruses, namely Sindbis (SIN) and Getah (GET) viruses; four are flaviviruses, namely West Nile (WN), dengue type 2 (DEN2), European tick-borne encephalitis

Table 1. Characterization of the viruses and methods of haemagglutinin preparation

Family	Genus	Species	Strain	Source	Method of haemagglutinin preparation
Togaviridae	Alphavirus	Sindbis	Eg Ar 339	Le Pharo, Marseille	SA[1]
		Getah	M 603	Pasteur Institute, Cambodia	SA + SP[2]
	Flavivirus	West Nile	K 99	Institute of Virology, Bratislava	SA
		Dengue 2	New Guinea B	Y.A.R.U., U.S.A.	SA
		European tick-borne encephalitis	Hypr	Institute of Virology, Bratislava	F[3]
		Wesselsbron	Onderspoort Gen 3	N.I.M.R., London	SA
Bunyaviridae	Bunyavirus	Čalovo	Original	Institute of Virology Bratislava	SA
		Ťahyňa	Original	Institute of Virology Bratislava	F + SP
	Uukuvirus	Uukuniemi	S 23	Y.A.R.U., U.S.A.	SA
	Bunyavirus-like	Bhanja	IG 690	Y.A.R.U., U.S.A.	SA

[1]SA = Sucrose-acetone
[2]SA + SP = Further treatment by protamin sulfate
[3]F = Freon 113

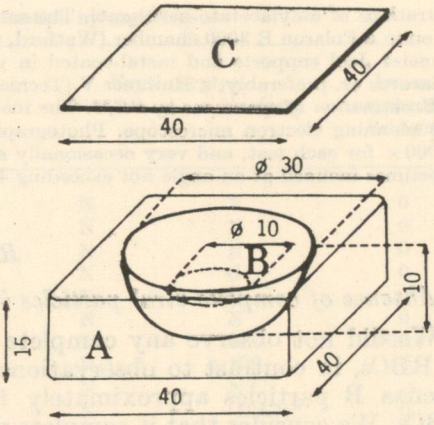

Fig. 1.

Haemagglutination chamber (sizes in mm)
A: salt-cellar with an almost flat--bottomed cup.
B: circular object-slide 10 mm in diameter placed at the base of the cup.
C: 40 × 40 mm glass slide 1 mm thick assigned to prevent drying-out of the reaction

(TBE) and Wesselsbron (WSL). Four viruses listed in Table 1 belong to the family Bunyaviridae. These comprise: two bunyaviruses, namely Čalovo (CVO) (=Batai), and Ťahyňa (TAH)-(=Lumbo), one is an Uukunivirus, Uukuniemi (UUK), and one is bunyavirus like, Bhanja (BHA). The characteristics and source of these strains, as well as the methods of haemagglutinin preparation are described in the same table.

For each virus the optimal and the unfavourable pH for haemagglutination were determined by microtitration in the range from 5.8 to 7.0.

Construction of a new type of haemagglutination chamber for the preparation of samples for SEM. The paper of Tawara *et al.* (1976) states that the haemagglutination reactions were carried out in tubes, the agglutinated RBC's present at the bottom of the tube being first fixed and then subjected to appropriate treatment for SEM examination. The authors, however, did not indicate how they treated the samples, and in particular, how were the samples metal-coated. There would appear to be no other way of doing this, unless breaking the tube and treating the fragments. This approach seemed to be unsatisfactory to us as arboviruses are potentially dangerous to handle; it also appeared liable to introduce artefacts into the specimens.

We, therefore, designed a sterilisable and re-usable haemagglutination chamber in the form of a broad and almost flat-bottomed cup, into which an object slide is placed: the agglutinates settle on to this slide, which can then be easily removed from the chamber and appropriately treated.

The chamber was made from a square glass salt-cellar measuring 40 × 40 mm and 15 mm thick (Fig. 1). It was carved to the shape of a broad U-shaped cup 30 mm in diameter and 10 mm deep. This is covered by a 40 × 40 mm glass slide 1 mm thick to prevent drying. A circular object-slide 10 mm in diameter is placed at the base of the cup. The haemagglutination reaction takes place in this chamber, which must not be moved during the process.

Haemagglutination. Each virus was tested simultaneously in three chambers as follows:

— haemagglutination test using 4 haemagglutination units at optimal pH and temperature;
— haemagglutination test using 4 units at the same temperature but at unfavourable pH, i. e. a pH outside the range seen in previous microtitrations;
— control test comprising only RBCs at temperature and pH optimal for the corresponding haemagglutinin.

The RBCs used came from 24 hr old chicks. The buffers were those of Clarke and Casals (1958).

Treatment of specimens destined for SEM. Once the agglutinated or non-agglutinated RBCs had settled, i. e. 2 to 3 hr after setting up the reaction, the buffer was carefully aspirated and replaced by a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer pH 7.0. Fixation was carried out for 3 days, after which the fixative was removed by aspiration. The specimen was then washed 3 times in distilled water, and rehydrated by passing through a series of baths of progressively increasing concentrations of ethanol in distilled water, followed by increasing con-

centrations of amylacetate in ethanol. The samples were then treated by critical-point evaporation in a Polaron E 3000 chamber (Watford, Great Britain). They were then stuck onto 10 mm diameter Jeol supports and metal-coated in vacuum with gold-palladium in an Edwards 306 apparatus or, preferably, a Hummer V (Tecnics EMS, Inc, U.S.A.).

Examination of specimens by SEM. The metal-coated specimens were examined in Jeol type P15 scanning electron microscope. Photographs were made at usual enlargements of 30× to 10.000× for each test, and very occasionally at 20.000×. The specimen was held horizontal or sometimes inclined at an angle not exceeding 40°.

Results

Absence of complete viral particles in the specimens

We did not observe any complete viral particles adsorbed to the surface of RBCs, in contrast to observations of Tawara *et al.* (1976) who found influenza B particles approximately 100—120 nm in diameter, attached to RBCs. We consider that if complete viral particles of the family Togaviridae (45—65 nm in diameter), or more particularly of the family Bunyaviridae (mean diameter 100 nm) had been attached to RBCs in our specimens and had been responsible for the formation of agglutinates, they could not have escaped our notice because of the range of the magnifications used.

Relatively uniform appearance of the pictures observed

At low magnification regardless to the haemagglutinin involved, the agglutinated RBCs were arranged in more or less well-structured clumps, separated one from another by "clefts" containing no RBCs. The shape and surface of the RBCs appeared grossly normal. The control reactions revealed neither structured clumps of RBCs nor clefts. At higher magnification, when the specimens were tilted during examination, some differences were observed which are summarized in Table 2 for family Togaviridae and in Table 3 for family Bunyaviridae.

Appearance of RBCs agglutinated by togaviruses in SEM

In the presence of haemagglutinin and at optimal pH, all viruses except of Getah (whose haemagglutination titre is low) caused formation of clumps of RBCs which were structured, i. e. some of them oriented on their edges rather than being just piled one on the top of another. The structured clumps were built up, at least in part, in a manner of a house of cards. They were separated one from another by definite clefts (Figs. 2 and 3). Platelets may have been found trapped in clumps of RBCs as for example with WN. At high magnification it can be seen that the shape and surface of the RBCs are perfectly preserved. However, with DEN2 virus, star-shaped figures with 5 or 6 branches can be seen applied to the surface of certain RBCs (Fig. 4). With TBE and GET viruses, under the same conditions, some granular structures could be observed, but these did not have the regularity of the star-shaped figures.

At unfavorable pH two viruses WN and DEN2 also produced structured clumps. Both of these revealed the highest haemagglutinating titres (1280)

Table 2. Haemagglutination by togaviruses

Virus (HA titre)		pH	Structured clumps	"Clefts" between clumps	Erythrocytes Shape	Erythrocytes Surface	Inter-erythrocytic bridges
SIN	I	6.2	+	+	N	N	0
(640)	II	6.8	0	0	N	N	0
	III	6.2	0	0	N	N	0
GET	I	6.0	±	±	N	G	0
(12)	II	6.8	0	0	N	G	0
	III	6.0	0	0	N	N	0
WN	I	6.5	+	+	N	N	Rare
(1280)	II	5.8	+	+	RD	NT	0
	III	6.5	0	0	N	NT	+
DEN2	I	6.3	+	+	N	(HA?)*	0
(1280)	II	6.8	+	+	N	N	0
	III	6.3	0	0	RD	Plicate	0
TBE	I	6.3	+	+	N	G	0
(80)	II	6.8	0	0	N	N	0
	III	6.3	0	0	N	N	0
WSL	I	6.0	+	+	N	N	0
(640)	II	6.8	±	0	N	N	0
	III	6.0	0	0	N	N	0

pH: I = Optimal; II = Unfavourable; III = Control test without virus;
G = Granulations; N = Normal; NT = Normal thromobocytes; RD = Rare deformations;
* Star-shaped figures; HA titre = haemagglutination titre; HA = haemagglutinin

(Fig. 5). The clumps were separated by definite clefts. Other viruses did not produce structured clumps, the RBCs being simply piled one on another. With WN virus, platelets were included in the RBC clumps. In control samples lacking haemagglutinin, the RBCs lay flat on ground surface and did not form clumps (Fig. 6).

Appearance of RBCs agglutinated by bunyaviruses in SEM.

In the presence of haemagglutinin and at optimal pH, all viruses studied produced structured clumps, some of these being very marked as with the UUK virus (Fig. 7) and separated by clefts. These, hovever, were less clear (especially by CVO and TAH viruses) and similar to what was seen with the GET virus. At high magnification, the shape and surface of the RBCs appeared perfectly preserved: no structures comparable to the star-shaped figures seen with DEN2 virus were observed. With BHA virus, inter-erythrocytic bridges and coarse granulations on the RBC surface were observed.

In the presence of haemagglutinin and at unfavourable pH, some clumps were visible but these were much less structured than at optimal pH. This was the case especially with TAH and UUK viruses. With BHA virus, inter-erythrocytic bridges and coarse granulations on the RBC surface were observed as they were seen at optimal pH. In the control samples, the RBCs

Table 3. Haemagglutination by bunyaviruses

Virus (HA titre)	pH Optimal	pH Unfavourable	pH Control test	Structured clumps	"Clefts" between clumps	Erythrocytes Shape	Erythrocytes Surface	Inter-erythrocytic bridges
	6.4			±	±	N	N	0
TAH (24)		5.8		±	0	N	N	0
			6.4	0	0	I	I	+
CVO (32)	6.4			+	±	N	N	0
		5.8		0	0	N	N	0
			6.4	0	0	N	N	0
	6.8			+	+	N	N	0
UUK (32)		5.8		±	±	N	N	0
			6.8	0	0	N	N	0
	6.4			+	+	N	CG	+
BHA (32)		5.8		0	0	N	CG	+
			6.4	0	0	N	N	0

N = normal
I = irregular
CG = coarse granulations
HA titre = haemagglutination titre

never formed clumps; they settled flat on the ground surface. The shape and surface of the RBCs were normal, except in the control sample for TAH virus, in which some deformation was seen.

Discussion

It is clear that the structured clumps separated by marked clefts correspond to agglutinates of RBCs which have settled on the slide after sedimentation. The nonagglutinated RBCs sediment separately and settle flat on the ground surface, forming no clumps or clefts.

No virus particles were found adsorbed to the RBCs surface and we did not find any complete virus particles in our specimens. It can be accepted that treatment with sucrose-acetone or Freon 113 caused the virions to burst liberating haemagglutinating subunits.

Only in the case of DEN2 virus at optimal pH, star-shaped figures were seen adhering to the RBC surface. Whether these figures are able to cause haemagglutination is a matter of conjecture. However the purified haemagglutinin subunits from influenza A virus also give rise to such star-shaped figures (Laver and Valentine, 1963). In a similar manner, the purified surface projections stripped off from the envelope of Semliki Forest virus (SF) by Triton X-100, formed soluble octameric complexes which, after negative staining, revealed the appearance of "rosettes" approximately 19 nm in diameter (Helenius and von Bonsdorff, 1976). Such complexes showed haemagglutinating activity and were capable to attache to corresponding cellular receptors (Helenius and von Bonsdorff, 1976).

Unfortunately, we neither know the exact morphology of the haemagglutinin of flaviviruses nor in particular that of DEN2. With whole virions, examined in section, the zone of surface projections were blurred and very ill-defined (Murphy, 1980). These projections are present sometimes in a form of rings 7 nm in diameter (Smith *et al.*, 1970) or sometimes in a form of spherical structures 7 nm in diameter (Demsey *et al.*, 1974). The exact nature of these star-shaped elements described above is still conjectural, but if these were compared to the "rosettes" of SF virus, this would imply that the haemagglutinins of alphaviruses and those of flaviviruses were similar in structure.

A further aspect of our results that merits discussion is the presence of clumps of RBCs, sometimes well-structured and separated by definite clefts, even when the haemagglutinin acted at unfavourable pH. In such experimental conditions, there should be no haemagglutination, yet SEM revealed the presence of agglutinates. We, therefore, think, that even at unfavourable pH values lattices of RBCs are formed. The experiments of Mooney *et al.* (1975) have shown, furthermore, that at a pH unfavourable for haemagglutination, SIN virus becomes fixed, at least in part, to liposomes derived from sheep RBCs. Thus agglutinates may form, but they must disintegrate more or less rapidly depending on the virus species; in certain cases, as with SIN, GET, TBE, CVO and BHA, the agglutinates must have been already destroyed at the time they became fixed in glutaraldehyde.

In other cases, as with WN and DEN2, the agglutinates remain compact and it will once again be noted that these are the viruses with the most powerful agglutinins (titre = 1280). Finally, other viruses, such as WSL, TAH and UUK, form porly structured agglutinates when conditions are unfavourable, or at least less well structured than when the pH is optimal; it is resonable to suppose that these agglutinates are in the process of disintegration at the time they are fixed.

In conclusion, the impression was gained that the mechanism of haemagglutination with viruses of family Togaviridae and Bunyaviridae is not fundamentally different. At all events, even though we remain ignorant of the nature of this process, the pictures produced by SEM are very similar. For example, haemagglutination brought about by GET virus (low titre haemagglutinin) is similar to that of caused by bunyaviruses which all have weak haemagglutinins (titres ranging from 24 to 32). It was not our aim to determine the precise mechanism of arbovirus haemagglutination by SEM, but we can nevertheless state that haemagglutination caused by viruses of both families (Togaviridae and Bunyaviridae) seemed not to depend on the presence of complete viral particles. The star-shaped structures may correspond to haemagglutinating subunits of DEN2 virus that have been reconstituted and adhere to the RBC surface. They, however, are too small in number to produce a lattice capable of aggregating RBCs.

The mechanism of haemagglutination by arboviruses may differ fundamentally from that of the myxoviruses. It is known that the haemagglutinins of alphaviruses, certain flaviviruses and certain bunyaviruses also have haemolytic properties (Karabatsos, 1963, 1965; Yoshinaka *et al.*, 1979; Väänänen and Kääriäinen, 1979, 1980; Yamamoto *et al.*, 1981). The fixation of haemagglutinating subunits on globular receptors, known to be lipid in nature (Salminen, 1962; Nicoli, 1965; Mooney *et al.*, 1975), might produce microlesions capable on their own of causing first aggregation and then sedimentation of damaged RBCs. This hypothesis might be studied by a combination of the use of highly purified haemagglutinins, transmission electron microscopy and SEM. This last technique should also enable us to explore the phenomenon of haemagglutination caused by other viruses: enteroviruses, adenoviruses, caliciviruses, reoviruses, rhabdoviruses, etc.

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Explanation of Electron Micrographs (Plates XXIX–XXXI):

Fig. 2. Haemagglutination by WN virus at optimal pH; clumps of RBCs separated by very clear clefts; × 530.

Fig. 3. Haemagglutination by WN virus at optimal pH; clumps of RBCs were structured in the manner of a house of cards; × 4000.

Fig. 4. Haemagglutination by DEN2 virus at optimal pH; star-shaped figures (*) applied on the surface of an agglutinated RBC. The specimen was held at 45°; × 30 000.

Fig. 5. Haemagglutination by WN virus at unfavourable pH; despite of haemagglutination at unfavourable pH, many clumps of RBCs occurred and were separated by distinct clefts; × 500.

Fig. 6. Control lacking haemagglutinin. The RBCs lay flat on the surface and did not form clumps or clefts. Furthermore, some RBCs are probably eliminated during processing of the specimen; × 530.

Fig. 7. UUK haemagglutination at optimal pH; structured clumps of RBCs are clearly separated by clefts; × 380.