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Simian Enterovirus SV2 Hemagglutination Studies. I. Relationship of Infectivity to Hemagglutination.* (29783)

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While studying the characteristics of a number of simian enteroviruses it was noted that SV2 agglutinated rhesus monkey red blood cells (RBC) at 4°C and room temperature but not at 37°C(1). To determine the nature of the hemagglutinin produced by this virus, a study was made of the relationship of the infectious virus particle to the hemagglutinating activity of the virus. The results have shown that the two are very closely associated and that the hemagglutinating activity probably resides in the viral capsid.

Materials and methods. Virus. The virus was obtained from the American Type Culture Collection, Viral and Rickettsial Registry and plaque purified in LLCMK2 cells. This virus served as a source for passages in primary rhesus monkey kidney cultures. These passages were used during this study. To increase the hemagglutination (HA) titer of the infected culture fluids (usually 32 to 128) the latter were concentrated on several occasions by dialysis against polyvinyl pyrrolidone after homogenization with genetron 113 (2 parts virus : 1 part genetron).

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Tissue culture. Primary rhesus kidney cell cultures were grown from suspensions of trypsinized kidney cells in Melnick's lactalbumin hydrolysate-Hanks' saline medium(2) containing 2% calf serum and 0.2% anti-SV5 rabbit serum (Microbiological Associates, Inc.). Bottle cultures were maintained on lactalbumin hydrolysate-Earle's saline medium(2) containing 2% calf serum. Tube cultures were maintained on 50% 199 medium-50% bovine amniotic fluid.

Virus assay. Infectivity titrations were carried out in tube cultures of primary rhesus kidney cells by inoculating 0.1 ml of 10-fold dilutions of the virus into each of 4 tubes per dilution. The cultures were incubated at 37°C and observed daily for 1 week. Infectivity endpoints ($TCID_{50}$) were determined by the method of Reed and Muench(3).

HA test. Rhesus monkey RBC were collected in Alsever's solution. These cells were washed 3 times with phosphate buffered saline (PBS) and used as a 0.5% suspension in PBS. Cells not used immediately were stored in Alsever's solution at 4°C for no longer than 1 week. A mixture of 0.5 ml RBC and 0.5 ml of 2-fold dilutions of virus in 13 × 100 mm glass tubes was used in the

test. These mixtures were incubated at room temperature for 2 hours followed by overnight incubation at 4°C. Tubes were read at 2 hours and after overnight incubation. The endpoints from these 2 readings were generally quite similar. Endpoints are expressed as reciprocals of the highest dilution showing 3-4+ hemagglutination by pattern formation.

Growth curve. The rate of growth of the virus was determined by inoculating monolayers of primary monkey kidney cells in 1 oz prescription bottles with 0.4 ml of a virus suspension at a multiplicity greater than 10. After 1 hour at room temperature the unadsorbed virus was washed off and the infected cells overlaid with 4 ml of Earle's saline containing 2% calf serum. At hourly intervals duplicate cultures were withdrawn for virus assay. The overlay was poured off and centrifuged. The supernatant fluid was collected for extracellular virus infectivity and HA determinations, and the sediment was added to the cells remaining in the bottles. The cells were then frozen and thawed for intracellular virus infectivity and HA determinations.

Density gradient. Buoyant density measurements were made by layering 1 ml of purified and concentrated virus over 3.5 ml of cesium chloride (40% w/w) followed by centrifugation at 30,000 rpm for 23 hours in the Spinco model L centrifuge (SW39 rotor). After puncturing the bottom of the tube with a 25 gauge needle, 3 drop fractions were collected and titrated for HA and infectivity.

Thermal inactivation. Aliquots (0.7 ml) of virus were distributed in 4 ml screw cap vials and submerged in a 45°C water bath for various time intervals. They were then cooled on ice and titrated for HA and infectivity.

Ultraviolet light (U.V.) inactivation. Ten milliliters of a virus suspension, which had been diluted 1:5 in PBS, were placed in a 100 mm glass petri dish bottom and exposed to a 15 watt General Electric germicidal lamp at a distance of 50 cm (approx. 12 ergs/sec/mm^2). Samples were taken at intervals for HA and infectivity titrations.

Photoinactivation. Virus grown in Earle's saline containing 2% calf serum and 4 μg/ml neutral red was dispensed in 1 ml amounts in new 13 $\times$ 100 mm glass tubes and exposed to 2-15 watt daylight fluorescent lights at a distance of 5 cm. HA and infectivity were determined at various intervals.

Adsorption of virus to RBC. Replicate mixtures of 0.1 ml virus and 0.1 ml packed rhesus RBC in 1 ml PBS were made at 4°C, room temperature and 37°C. At various intervals the mixtures were centrifuged to sediment the RBC, and HA and infectivity titrations were done on the supernatant fluid. Centrifugations were done at the temperature of incubation.

Results. Growth studies. When a one-step growth curve was obtained for SV2 it was found that there was a 3- to 4-hour eclipse period before infective intracellular virus appeared and that this intracellular synthesis was nearly complete in 3 hours (Fig. 1). Cytopathology was first noted at this time (6 hours). Extracellular infective virus began to appear during the 7th hour after inoculation or at about the time intracellular virus was approaching maximum concentration. Most of the virus remained associated with the cell during the 24-hour observation period.

The HA activity of these cultures first appeared in the cell at 5 hours and increased to a maximum 2 hours later. Extracellular HA activity appeared sometime between the 12th and 18th hour. Again, most of the hemagglutinin remained associated with the cell.

Buoyant density determination. When SV2 was centrifuged in a cesium chloride density gradient, the infectious virus particle and the hemagglutinin were found to have the same buoyant density (Fig. 2).

Thermal inactivation. When SV2 virus was heated at 45°C the infectivity and HA activity were inactivated quite rapidly and at the same rate (Fig. 3). Further, if the virus suspension was made molar in regard to magnesium chloride, it could be heated at 50°C for 1 hour without losing infectivity or HA activity (Table I).

U.V. irradiation and photoinactivation.

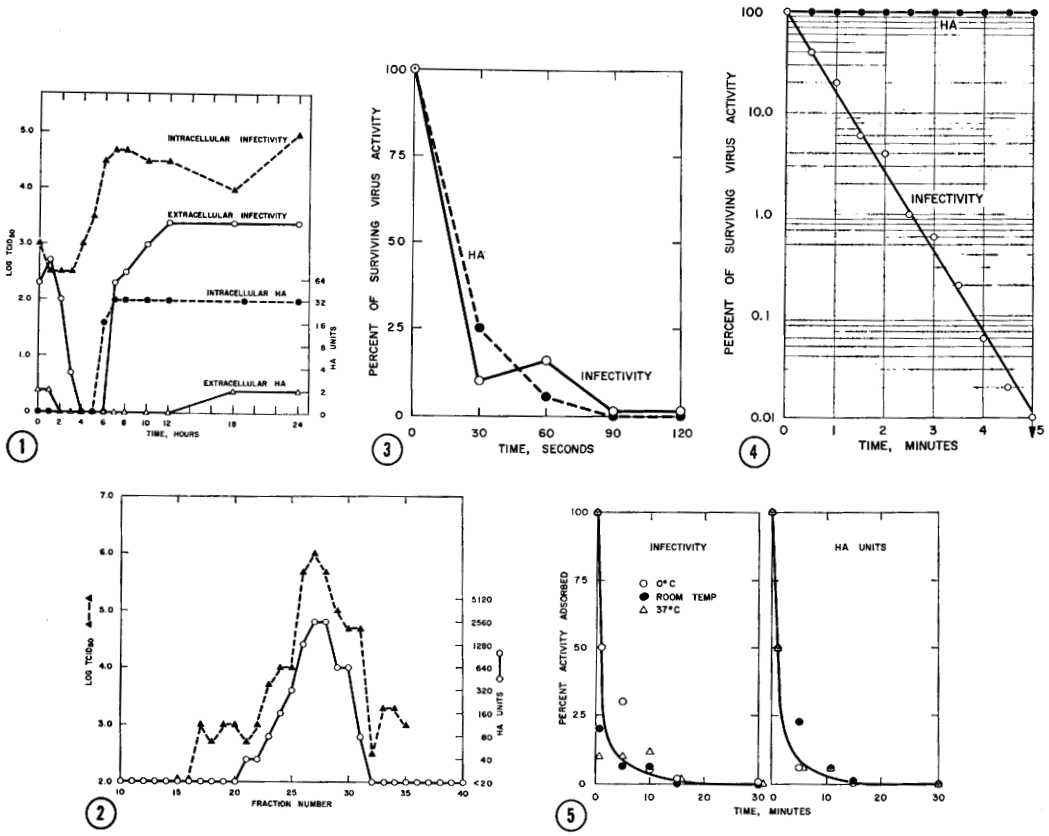

FIG. 1. Rate of appearance of extra- and intracellular hemagglutinin and infectious virus.
FIG. 2. Sedimentation of hemagglutinin and infectious virus in a cesium chloride density gradient (40% w/w).
FIG. 3. Rate of thermal inactivation of hemagglutinin and infectivity at 45°C.
FIG. 4. Rate of ultraviolet light inactivation of hemagglutinin and infectivity.
FIG. 5. Rate of adsorption of hemagglutinin and infectious virus to rhesus RBC at 0°C, room temperature and 37°C.

When the virus was irradiated with ultraviolet light it quickly lost its infectivity. The HA activity on the other hand remained unaffected for at least 15 minutes, long after all infectivity was destroyed (Fig. 4). SV2 virus, sensitized by growth in the presence of neutral red, also exhibited a rapid decrease in infectivity when irradiated with white light,

TABLE I. Stabilization of Infectivity and HA Activity by M Mg Cl_2.

Treatment	H_2O		M Mg Cl_2	
	Infectivity	HA	Infectivity	HA
Titer at 0 time	5.0*	32	5.0	32
" after 1 hr at 50°C	0	<2	5.0	32

* Log $TCID_{50}$/0.1 ml.

TABLE II. Effect of Photoinactivation on Infectivity and HA Activity.

Time of exposure (min)	Infectivity	HA
0	3.7*	64
5	2.0	64
15	2.3	64
30	2.0	64
45	2.0	64
60	2.0	32
Virus kept in dark 60 min	4.0	32

* Log $TCID_{50}$/0.1 ml.

while retaining the ability to agglutinate RBC (Table II).

Adsorption to RBC. When SV2 virus and rhesus RBC were mixed together at 4°C, room temperature and 37°C infectivity and

HA activity adsorbed at aproximately the same rate and rate of adsorption was not affected by changes in temperature (Fig. 5).

Discussion. It is well known that enteroviruses differ in their ability to agglutinate RBC. These differences can be used as a means of simplifying virus identification. It is also possible that comparative studies on agglutinating and non-agglutinating viruses will yield information on the function of some of the structural components of the virus particle in regard to attachment of the virus to cell receptors.

Hull *et al*(4) were the first to report that SV2 agglutinates rhesus RBC at 5°C. Subsequently, we showed that the agglutination occurs at 4°C and RT but not at 37°C and that only simian RBC were agglutinated(1). This latter observation correlates with that of Philipson and Bengtsson(5) who showed that human enteroviruses agglutinate only human RBC.

The growth studies presented here show that the SV2 viral hemagglutinin appears after the infective particle is assembled and for the most part remains intracellular, as does the infectious unit. These data suggest that the hemagglutinin is closely associated with the intact, infectious viral particle. Further evidence for this is that the infectivity and HA activity have the same buoyant density and adsorb to RBC at the same rate. The latter phenomenon indicates the adsorption to RBC is an electrostatic process similar to that found for other enteroviruses when they adsorb to cell cultures(6). An unexpected finding was that SV2 adsorbed to RBC equally well at 4°C and 37°C yet failed to agglutinate these cells at 37°C. Preliminary experiments indicate that this is not due to rapid elution because hemagglutinin has been observed to remain bound to RBC at 37°C for several days (unpublished data).

The data obtained for the effects of ultraviolet light and photodynamic inactivation show that infectivity can be destroyed without damaging the hemagglutinin. This suggests that inactivation of the nucleic acid core of the virus does not affect hemagglutinating activity and, therefore, the hemagglutinin resides in the viral capsid. On the other hand, preliminary experiments carried out by us show that photo- and U.V.-inactivated virus loses its ability to adsorb to primary rhesus monkey kidney cells while retaining the ability to agglutinate RBC (unpublished data). Further studies are being done on the relative rates of inactivation of the nucleic acid core and protein coat of SV2 by these agents.

Summary. The hemagglutinating activity for the simian enterovirus SV2 has been shown to be closely related to the infectious virus particle. The time of its appearance in the cell and the period required to reach maximum titer parallel the synthesis of infectious virus. Furthermore, it has the same buoyant density as infectious virus and adsorbs to RBC at the same rate. Exposure to ultraviolet light, and to white light in the presence of neutral red, destroys infectivity without affecting the hemagglutinin. Both properties are quickly destroyed by heat.

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