Hemagglutinin of Rabies and Some Other Bullet-Shaped Viruses (32864)

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The presence on rabies virus particles of surface projections morphologically similar to those on myxoviruses suggests the possibility that hemagglutinin activity may occur (1). Techniques recently developed in this laboratory for the preparation of rubella virus hemagglutinin in serum free suspension culture of BHK-21 cells (2) and for optimal titration of rubella virus hemagglutinin (3) were applied in an attempt to demonstrate this property for rabies virus and other bullet-shaped viruses of animals.

Materials and Methods. Viruses. The rabies virus strain used was CVS 11 at the one hundred-twelfth passage in primary hamster kidney cell culture (4). The infectivity titer of this seed virus was $10^{5.4}LD_{50}$/0.03 ml. Infected suckling mouse brain suspensions of prototype strains of other bullet-shaped animal viruses were used: these were vesicular stomatitis virus (VSV)-Indiana serotype ($10^{9.3}$ pfu/ml or $TCID_{50}$/ml in BHK-21 cells), VSV-New Jersey serotype ($10^{9.2}$) Cocal ($10^{9.6}$), Kern Canyon ($10^{7.5}$) and Flanders ($10^{4.3}$).

Cell cultures.[2] Preparation of suspension cultures of BHK-21/13S cells has been described in detail elsewhere (5). About 5×10^8 cells from 3 to 4 day old monolayer cultures were suspended in 800 ml of BHK-21 medium with 10% tryptose phosphate broth (6,7) and 10% heat inactivated fetal calf serum (FCS). After growth in suspension for one day at 37°C, cells were sedimented by low speed centrifugation and resuspended in BHK-21 medium with 0.4% bovine albumin (fraction V, reagent for microbiological use, Armour Pharmaceutical Company, Kankakee, Illinois). This medium is referred to as albumin medium.

Virus inoculation and harvest. The BHK-21 cells in 100 ml of albumin medium were inoculated with 15 ml of undiluted rabies virus stock. After 1 hour of incubation at 35°C with occasional shaking, 700 ml of the albumin medium was added. After overnight incubation at 35°C with constant spinning, the cells were sedimented again by low speed centrifugation and resuspended in 800 ml of fresh albumin medium. Incubation was continued at 35°C with spinning for various time periods; the pH was adjusted daily to 7.0–7.2 with 5% $NaHCO_3$. The cultures were centrifuged at 2000 rpm for 10 min and the supernatants were used as hemagglutinating antigens.

Hemagglutination and hemagglutination inhibition tests. Hemagglutination (HA) and hemagglutination inhibition (HI) titrations were performed in disposable microtiter U-plates (Linbro Chemical Co., Inc., New Haven, Conn.) by a slight modification of a technique commonly used for arboviruses (3, 8). The antigen and serum diluent was 0.4% bovine albumin–borate saline, pH 9.0. Final pH levels of 5.8–6.8 were achieved by suspending erythrocytes in appropriately buffered phosphate saline solutions (8). The volume of antigen dilutions used in titrations was 0.025 ml. An equal volume of diluent was added, followed by 0.05 ml of a 0.25% erythrocyte suspension. The latter was added while the plates were agitated on a reciprocating vibrator. Plates were sealed with tape and immediately placed on an ice slurry for development of the hemagglutination pattern. Patterns were recorded after 40–50 min of incubation with goose cells, or 1–1.5 hours of incubation with cells of other species. The end point was considered the highest dilution of antigen causing complete agglutination.

In the HI test, the volume of serum and

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[2] Use of trade names is for identification only and does not constitute endorsement by the Public Health Service of the U. S. Department of Health, Education, and Welfare.

antigen dilutions was 0.025 ml. The antigen was diluted to contain 2 hemagglutinating units per 0.025 ml. After preincubation of the serum–antigen mixtures for 2 hours at room temperature, the reagents were cooled on an ice slurry and 0.05 ml of the erythrocyte suspension was added to each well. The plates were incubated, and the test was recorded as indicated above. The highest initial serum dilution causing complete inhibition of agglutination was taken as the antibody titer.

Infectivity titrations. Infectivity titrations of rabies virus were carried out by intracerebral inoculation of 3-week-old mice. The titers are expressed as LD_{50}/0.03 ml.

Treatment with β-propiolactone. Rabies hemagglutinin was treated with β-propiolactone (BPL) according to the method of Sever *et al.* (9). The pH was adjusted with 0.2 *N* NaOH several times during the treatment.

Cytological procedures. For staining with acridine orange (AO) or with fluorescein isothiocyanate conjugated rabies antibody (FA), cells from suspension cultures were washed in Hanks' solution, dispersed on slides, air dried, and fixed for 1 hour in acetone (FA) or 10 min in methanol (AO). The staining method has been described elsewhere (4).

Results. Rabies Hemagglutinin. The development of infectious rabies virus and rabies hemagglutinin in duplicate suspension cultures of BHK-21 cells, one maintained in albumin medium, the other in the same medium with 6% FCS substituted for the albumin, is shown in Table I. In the albumin medium, peak infectivity of $10^6 LD_{50}$/0.03 ml occurred 2 days after virus inoculation and peak HA titer of 1:32 per 0.025 ml occurred on the sixth day. In the medium containing FCS the highest infectivity ($10^{5.8} LD_{50}$/0.03 ml) was obtained on the fifth day and HA activity was not detected in any of the samples.

Samples of infected and control cells were stained at intervals with AO or FA. Infected cultures in albumin medium showed progressively increased amounts of intracytoplasmic specific rabies antigen beginning with dust-like particles which were apparent 24 hours after inoculation. Eighty to 90% of the cells contained viral antigen at 48 hours. By the third day, large masses of material staining with FA were observed in the cytoplasm and also extracellularly. Fluorescing red cytoplasmic masses observed in preparations stained with AO paralleled in amount, size, and location those antigenic masses observed by FA. Infected cultures in FCS developed cytoplasmic changes at a slower rate and practically no extracellular antigenic material was demonstrated. Uninoculated cultures developed no comparable changes demonstrable by FA or AO staining.

TABLE I. Development of Infectious Rabies Virus and Hemagglutinin in Duplicate Suspension Cultures of BHK-21/13 S Cells Maintained in Albumin Medium and in Medium Containing 6% Fetal Calf Serum.

Medium	Days after virus inoculation	LD_{50} per 0.03 ml (log units)	HA titer per 0.025 ml (reciprocal)
0.4% bovine albumin, fraction V	0	2.8	0
	1	3.4	0
	2	6.2	0
	3	6.0	2
	4	5.6	8
	5	5.0	16
	6	3.6	32
6% fetal calf serum	0	1.5	0
	1	2.3	0
	2	4.8	0
	3	4.3	0
	4	5.3	0
	5	5.8	0

Optimal conditions for demonstration of rabies hemagglutinin included a low temperature, pH 6.2 (Table II) and the use of goose erythrocytes. Hemagglutination was not observed at room temperature or at 36°C. The positive hemagglutination pattern once fully developed in ice slurry incubation rapidly reverted to negative at room temperature. The pattern started to break down in 50–60 min in the higher dilutions even in the ice slurry.

Erythrocytes of other species were tested at various pH levels for sensitivity to rabies HA. All were agglutinated, but HA titers were only 1:1 to 1:4 with the erythrocytes of adult chickens, guinea pigs, rats, sheep, and human (type O); they were 1:8 to 1:16 with the erythrocytes of 2-day-old chicks, vervet, and rhesus monkeys. The comparable HA titer with goose cells was 1:32.

TABLE II. Rabies Hemagglutinin Titers at Various pH Levels Using 0.25% Suspension Goose Erythrocytes.

	Dilution of rabies hemagglutinin								
pH	Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
5.6	+	+	+	+	—	—	—	—	—
5.8	+	+	+	+	—	—	—	—	—
6.0	+	+	+	+	+	±	—	—	—
6.2	+	+	+	+	+	+	—	—	—
6.4	+	+	+	+	—	—	—	—	—
6.6	+	—	—	—	—	—	—	—	—

Effect of heat inactivation, β-propiolactone treatment, sonication and Tween 80–ether treatment on rabies hemagglutinin. The effect of heat inactivation at 56°C on rabies hemagglutinin and infectivity is shown in Table III.

The hemagglutinin was stored at +4, —20, and —70°C for 3 months with no decrease in titer. The hemagglutinin was also frozen and thawed 10 times without decrease in titer.

Treatment with 0.01–0.05% BPL did not change the titer of rabies hemagglutinin (Table IV), whereas infectivity was completely destroyed with each BPL concentration tested.

TABLE III. Inactivation of Rabies Hemagglutinin and Infectivity at 56°C.

Inactivation time (min)	Hemagglutinin titer (reciprocal)	Infectivity titer LD_{50}/0.03 ml (log units)
0	16	3.5
1	16	3.5
3	16	3.5
5	8	2.5
10	8	2.0
15	8	NT
20	8	1.3
25	4	NT
30	4	<1.0
45	2	NT
60	1	<1.0

TABLE IV. Effect of β-Propiolactone Treatment on Rabies Hemagglutinin and Infectivity.

β-Propiolactone concentration (%)	Hemagglutinin titer (reciprocal)	Infectivity titer LD_{50}/ml (log units)
0	16	3.5
0.01	16	0
0.025	16	0
0.05	16	0
0.1	8	0
0.2	0	0

Sonication of rabies hemagglutinin in a Raytheon sonic oscillator (model no. DF 101) at 10 kc for 1–10 min increased the HA titer twofold. After 20–60 min sonication the titer decreased to the original level.

Tween 80–ether treatment inactivated the hemagglutinin; no HA activity was recovered either from the water or ether phase. Alkaline extraction (3) of the packed infected cells harvested on the sixth day from the culture containing 6% FCS showed no activity.

Rabies hemagglutination inhibition. Individual normal human sera, pooled human serum, and normal burro, and goat sera contained high titered (1:10,000–1:40,000) nonspecific HA inhibitors which were difficult to remove. Methods used in attempts to remove these inhibitors included adsorption with kaolin, precipitation with cold acetone, treatment with RDE, trypsin and periodate, CO_2 and manganous–heparin. Only adsorption of diluted serum (1:10) with 25% kaolin at pH 9.0 (3) completely, or almost completely, removed nonspecific inhibitors detectable at a 1:20 serum dilution. This was tentatively adopted as a standard procedure realizing that it might also decrease specific antibody titers. The HI titers of antirabies hyperimmune sera prepared in burros and goats are indicated in Table V.

Human sera collected after rabies vaccination exhibited high titered goose hemagglutinin activity apparently produced by the duck tissue in the vaccine. To avoid this problem, chick erythrocytes were employed in

TABLE V. Homologous and Heterologous Hemagglutination Inhibition Titers of Rabies and Some Other Bullet-Shaped Viruses.

Antiserum	Viruses				
	Rabies	VSV-Indiana	VSV-New Jersey	Cocal	Kern Canyon
Rabies, burro no. 3[a]	320	<20	<20	<20	40
Rabies, burro no. 4[b]	320	<20	<20	<20	<20
Rabies, burro no. 5[c]	1280	<20	<20	<20	20
Rabies, goat no. 15[b]	160	<20	<20	<20	<20
Rabies, goat no. 19[c]	1280	<20	<20	<20	<20
Normal burro	<20	<20	<20	<20	<20
Normal goat	<20	<20	<20	<20	<20
VSV Indiana[d]	<20	80	<20	20	<20
VSV New Jersey[d]	<20	<20	160	<20	<20
Cocal[d]	<20	80	20	160	<20
Kern Canyon[d]	<20	<20	<20	<20	160
Flanders[d]	<20	<20	<20	<20	<20
Hart Park[d]	<20	<20	<20	<20	<20

[a] Prepared by immunizing with a commercial rabies vaccine for dogs (nervous tissue of caprine origin, inactivated).

[b] Prepared by immunizing with BHK-21 cell culture grown virus.

[c] Prepared by immunizing with ecteola-cellulose column purified virus grown in suckling mouse brain.

[d] Hyperimmune ascitic fluid of mice inoculated with mouse brain suspension of virus.

the HI test, or 1.0 ml of the 1:20 diluted kaolin treated serum was adsorbed twice with 0.2 ml of packed goose cells.

The HI titers of vaccinated persons varied from less than 1:20 to 1:320. However, titers of 1:20 were occasionally observed in the sera of nonvaccinated persons, suggesting incomplete removal of nonspecific inhibitors by the kaolin treatment.

Hemagglutinin and hemagglutination inhibition of some other bullet-shaped viruses. The methods described were used with minor modifications in the preparation of hemagglutinins of VSV-Indiana, VSV-New Jersey, Cocal, and Kern Canyon viruses. The modifications included the inoculation of 800 ml suspension cultures with 2 ml of 1:100 diluted stock virus suspensions and the changing of medium of the suspension culture a few hours after virus inoculation instead of the next day. The optimal harvest time was usually 2 days after virus inoculation. Cytopathogenic effect progressed rapidly thereafter. Using goose erythrocytes, the peak HA titer and pH optimum was 1:64 at pH 5.8 for VSV-Indiana, 1:32 at pH 6.4 with VSV-New Jersey, 1:16 at pH 6.2 with Cocal, and 1:128 at pH 6.2 with Kern Canyon virus. Erythrocytes from other species were not tested and in most of the HA titrations at various pH values, only one antigen batch was used. No hemagglutinin could be demonstrated with these techniques for Flanders virus and no clear cytopathogenic effect on BHK-21 cells was observed in 6 days, at which time a spontaneous degeneration occurred in control cells. Homologous and heterologous HI titers of VSV-Indiana, VSV-New Jersey, Cocal, and Kern Canyon viruses are shown in Table V.

Discussion. The results of the present study indicate that a hemagglutinin of rabies virus can be prepared in suspension culture of BHK-21/13 S cells. The negative HA results in earlier reports (4) can be explained by the special requirements both in preparation and demonstration of rabies hemagglutinin. These optimal conditions include an inhibitor free medium in suspension culture, a low temperature, pH of 6.2, and the use of goose erythrocytes. These requirements are somewhat similar to those of arboviruses, with the

exception of the more critical need for ice slurry temperature. Optimal conditions for rubella HA also include low temperature, but at room temperature the titers are only slightly lower. In addition, rubella hemagglutinin pattern once fully developed at ice slurry temperature is stable at room temperature and chick erythrocytes are more sensitive than goose erythrocytes. Rabies hemagglutinin patterns rapidly revert to negative at room temperature and goose erythrocytes are more sensitive than chick erythrocytes.

The effect of albumin medium on the growth of rabies virus in BHK-21 cells is of interest. In addition to its importance as an inhibitor free medium, apparently it enhances both adsorption and release of rabies virus from BHK-21 cells. The earlier appearance of viral antigen in infected cells maintained in albumin medium than in cells maintained in fetal calf serum medium is similar to the effect of polyions as demonstrated by Kaplan *et al.* (10).

Development of a sensitive HI test for rabies antibody titration would be highly desirable. However, more information on the nature of high titered nonspecific inhibitors is required for development of optimal techniques for removing these substances from serum without decreasing specific antibody activity. The rabies HA antigen in the crude form seems to be relatively insensitive to antibody. Modification of techniques such as the Tween 80–ether treatment which has been successful with measles and rubella HA antigens may render rabies HA antigen more suitable for the HI test.

No heterologous reactions were demonstrated between rabies HA antigen and hyperimmune mouse ascitic fluids of the other bullet-shaped animal viruses. Those heterologous HI reactions between other bullet-shaped viruses are similar to the results of Murphy and Fields (11) who used other immunologic methods to study the interrelationships in the group. Only the reactions of rabies burro sera number 3 and 4 with Kern Canyon hemagglutinin are anomalous. Whether these titers represent a true antigenic relationship, or a nonspecific reaction, cannot be determined from the present data.

Demonstration of similar hemagglutinins of rabies and four other bullet–shaped viruses suggests a common biological property consonant with described similarities in physical structure of this group of viruses. These and other bullet-shaped viruses are currently being brought together into the *Stomatoviridae* family primarily on the basis of common physical characteristics (11).

Summary. Hemagglutinin of rabies virus was prepared from CVS 11 strain grown in suspension culture of BHK-21 cells maintained in a medium containing 0.4% bovine albumin and no serum. Optimal conditions for demonstration of rabies hemagglutinin included low temperature, pH 6.2, and the use of goose erythrocytes. Normal human, burro, and goat sera contained high titered nonspecific HA inhibitors which were difficult to remove. Specificity of rabies HA antigen was, however, demonstrated with antirabies hyperimmune sera which were treated with a modified kaolin adsorption technique. The same methods were used with minor modifications in the preparation of hemagglutinins of VSV-Indiana, VSV-New Jersey, Cocal, and Kern Canyon viruses.

All rabies hyperimmune sera were kindly supplied by Dr. Richard E. Dierks, Rabies Investigations Laboratory, NCDC, Atlanta. The supervision of all tissue culture preparation by Mrs. Anna Hall and the excellent technical assistance of Miss Karen Caswell, Miss Marianne Davis and Miss Ludie Holland is gratefully acknowledged.

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Received Nov. 27, 1967. P.S.E.B.M., 1968, Vol. 127.

Fat Storage, De-etherification and Elimination of Quinestrol in the Rat as Influenced by Route of Administration (32865)

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Quinestrol (ethynylestradiol-3-cyclopentyl ether; EECPE) is a potent oral estrogen in animals and human beings (1–4). Unlike its parent compound, ethynylestradiol (EE), EECPE is stored in and slowly released from body fat and brain of rats (5, 6). Storage is enhanced when a lipid rather than an aqueous vehicle is used (7). That portion of the dose which is not stored is metabolized to glucuronide and sulfate conjugates and rapidly eliminated in the bile and urine (8, 9). Using doubly labeled EECPE, it was shown that considerable cleavage of the cyclopentyl ether linkage occurs with elimination of the cyclopentyl label in the urine in excess of the steroid label (8). The site of de-etherification was unknown but was presumed to be either the gut or the liver. If the gut were the major site of de-etherification, then iv administered EECPE should undergo very little cyclopentyl group cleavage. Similarly, if the liver were the major site of de-etherification, then EECPE administered ip or po should initially undergo more extensive de-etherification than that injected iv by virtue of being absorbed entirely via the hepatic portal system. In the present experiments, doubly-labeled EECPE was administered by various routes and fat storage and excretion of the label was followed in the hope of clarifying the site of removal of the cyclopentyl group. In addition, the excretion of radioactivity following administration of labeled cyclopentanol was compared with that following EECPE labeled in the cyclopentyl ring.

Material and Methods. A. General. The chromatographically homogeneous radiosteroids used were ethynylestradiol-6,7-^{3}H-3-cyclopentyl ether (specific activity 1 μC/μg) and ethynylestradiol-3-cyclopentyl-1′-^{14}C ether (specific activity 0.037 μC/μg).[1] Doses were in general prepared to contain ^{3}H and ^{14}C in a ratio of approximately 10:1. Specific doses are indicated in subsequent sections and in the tables. Cyclopentanol-1-^{14}C (New England Nuclear Lot No. 161-275A, specific activity 0.023 μC/μg) was administered at a dose of 0.1 μC (4.3 μg) in aqueous vehicle.

In designing the various experiments, it was assumed that the concentration of radioactivity in body fat 24 hours after dosing would be proportional to the body store of unmetabolized EECPE. This assumption is supported by previous data which showed that: EECPE is rapidly absorbed from the gut when administered as an aqueous suspension (7); it is stored unaltered in body fat and brain (6); its transformation products are not so stored (7, 9); the fat levels remain relatively constant from hours 8 to 48 (7).

B. Influence of dose and vehicle of administration on body fat and brain storage. These experiments were performed to determine if excessive doses of EECPE would retard absorption or overwhelm metabolic or storage sites. Variable amounts of unlabeled EECPE were added to a constant amount (8.5 μC) of tritiated material. Doses varying from 20 μg to 12.5 mg were administered orally either as oily solutions or aqueous suspensions to male albino rats 300–400 gm of body weight. The animals were killed 24 hours after administration and radioactivity in perirenal fat and brain extracted and determined

[1] Radioactive steroids synthesized by Mr. E. Merrill, Radioisotope Safety Officer, WLRI.