Nariva Virus: Further Studies, with Particular Reference to Its Hemadsorption and Hemagglutinating Properties (33680)

N. KARABATSOS, S. M. BUCKLEY, AND P. ARDOIN
(Introduced by J. Casals)

Yale Arbovirus Research Unit,[1] *Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510*

Nariva virus is an ether-sensitive agent, isolated exclusively from forest rodents, *Zygodontomys b. brevicauda*, in Trinidad and found to be unrelated antigenically to a large number of recognized arboviruses. In describing the virus, Tikasingh *et al.* noted that "The demonstrated ether sensitivity warrants consideration of this agent for inclusion as an arbovirus, but final resolution of this point must await further study" (1).

The investigations reported here concern biological and physical properties of the virus, and were prompted by the discovery that cell cultures infected with Nariva reacted positively in routine hemadsorption tests.

Materials and Methods. Virus and cell cultures. Fourth suckling-mouse brain passage Nariva virus, strain Tr 42520, was used. Virus stock was prepared as a 10% mouse brain suspension in phosphate-buffered saline (PBS), pH 7.2, containing 7.5% bovine albumin and was stored lyophilized at —20°.

The BHK-21 cell cultures were propagated, transferred, and maintained as described elsewhere (2). Stock cultures of Vero, HeLa, LLC-MK_2, PS-Y15, and MA-111 cells were grown in 90% Eagle's basal medium with 10% calf serum. All tube cultures were prepared and maintained as described for BHK-21 cells (2).

Inhibition of virus multiplication. The effect of 5-iododeoxyuridine (IUDR) on virus multiplication was studied in BHK-21 tube cultures by procedures outlined previously (3). Vaccinia virus (DNA) and eastern encephalitis (EE) virus (RNA) were employed as control viruses of known nucleic-acid composition. A 10^{-2} *M* stock of IUDR was prepared in distilled water; to achieve solubility, 0.1 *N* NaOH was added to raise the pH to 9.0 and the solution was heated briefly to 75°. Cultures were incubated for 4–5 hr with maintenance medium containing 10^{-4} *M* IUDR solution, drained free of this medium and inoculated with virus, and then refed with fresh maintenance medium containing IUDR. Fluids were changed daily. Non-IUDR-treated cultures served as controls. All cultures were harvested when the controls showed at least a 3+ cytopathic effect (CPE); Nariva-, EE-, and vaccinia-infected cultures were harvested 6 days, 3 days, and 24 hr postinoculation, respectively. The cells and fluid were frozen and thawed once and then assayed for virus in BHK-21 tube cultures using 5 tubes/dilution.

Antigens. Complement-fixing (CF) antigen for Nariva virus was prepared from infected mouse brains by sucrose–acetone extraction (4). In confirmation of the original report (1), this preparation did not agglutinate goose red blood cells (RBC). Hemagglutinin (HA) was prepared from Vero tube cultures 6–8 days after inoculation of a 10^{-1} or 10^{-2} dilution of Nariva stock virus. Infective fluids from 3 or 4 tubes were added to the fluid and still-adhering infected cells in a fourth or fifth tube, and the latter was sonicated with the Bronwill Biosonik II[2] while immersed in an ice-water bath. Sonication was performed for 45–60 sec, with constant varying of the intensity between minimum and maximum. The mixture was then transferred to one of the other tubes and again sonicated, and this process was repeated until all 4–5 tubes had been sonicated. The material was then sedimented in a Spinco ultracentrifuge at 35,000 rpm for 60 min, after which the pellets were resuspended in 8.5% sucrose containing 0.4% bovine albumin and the suspension was sonicated once again. The

[1] The Yale Arbovirus Research Unit is supported in part by The Rockefeller Foundation.

[2] Bronwill Scientific, Rochester, N. Y.

specificity of each sonicated HA preparation, and of those treated with Tween 80 and ether (TE) as described by Norrby (5), was checked in hemagglutination-inhibition (HI) test with homologous Nariva antiserum.

Immune reagents. Hyperimmune sera were prepared in adult mice given 3 injections of live virus at monthly intervals, the first dose consisting of 1% mouse brain suspension administered intracerebrally and the second and third of 10% mouse brain suspension given intraperitoneally (i.p.). Mice were bled 10 days after the last injection.

Immune ascitic fluid was prepared in adult mice by a modification of a previously described procedure (6). An i.p. injection of 10% mouse brain suspension mixed in equal quantities with incomplete Freund's adjuvant was given on days 1 and 8, and a booster dose on day 36. Sarcoma 180/TG (6) was given i.p. 1 week before the booster dose, and ascitic fluid was tapped 1 week after the booster dose. All serological studies performed with immune ascitic fluid could be referred to material from this single tapping interval.

CF tests. Varying dilutions of antigen (only 2 dilutions in a few instances) were tested in micro CF system against varying dilutions of serum or ascitic fluid, using 2 units of complement and overnight fixation at 4°.

Hemadsorption, hemagglutination, and HI tests. Hemadsorption testing (7) was done with freshly obtained guinea pig RBC. Unless otherwise specified, Nariva HA was assayed at 4° using 0.5% guinea pig RBC. Serial dilutions of the HA were made in 0.2-ml volumes of PBS diluent containing 0.4% bovine albumin, and the total volume of the test was 0.4 ml.

Treatment of guinea pig RBC with trypsin or receptor-destroying enzyme (RDE) was done as previously described (8), using 1 mg/ml of crystalline trypsin[3] and a 1:3 dilution of commercial RDE.[4] Untreated RBC were washed and kept as a stock 10% suspension in PBS.

In adsorption and elution experiments, Nariva HA and 2% guinea pig RBC were mixed in equal volumes and adsorption was allowed to occur for 90 min at 4°. The RBC were then sedimented in the cold, washed once with cold saline, resuspended to the original volume and held at 37°. Samples were taken at 1 and 2 hr, and the RBC were centrifuged at room temperature.

For HI tests (4), all sera and ascitic fluids were treated with kaolin to remove nonspecific inhibitors. Four HA units were mixed with serial dilutions of serum or ascitic fluid and incubated for 60 min at room temperature before the addition of 0.5% guinea pig RBC. The highest dilution showing complete inhibition was taken as the end point.

Results. CPE in cell lines. It was initially observed that Vero and BHK-21 cultures infected with stock Nariva virus developed syncytia and reacted positively in hemadsorption test with guinea pig RBC both at 4° and at room temperature. Syncytium formation was especially pronounced in BHK-21 cultures, eventually involving the entire cell sheet.

When Nariva virus as first-passage BHK-21 harvest was inoculated into these and other cell lines (Table I), syncytium formation was observed only in BHK-21 and Vero cultures. The CPE was minimal in HeLa and LLC-MK_2 cells and absent from PS and MA-111 cells. Viral multiplication was not apparent in the PS cultures, and probably none occurred in the MA-111 cultures; the infectivity detected by subinoculation into BHK-21 cultures presumably represents residual inoculum virus. With HeLa cells, hemadsorption testing and subinoculation detected slightly more virus than was indicated by direct observation of CPE.

Replication of Nariva virus in the presence of IUDR. The ability of halogenated deoxyuridine compounds to inhibit synthesis of DNA viruses (9–11) provides a means of differentiating the latter from RNA viruses. As 10^{-4} *M* IUDR did not reduce the yield of Nariva or of EE (the control RNA virus)

[3] Mann Research Laboratories, New York, N. Y.

[4] Berhingwerke, Marburg, Germany.

TABLE I. Susceptibility of Various Cell Lines to Infection with Nariva Virus (first passage in BHK-21 cultures).

Cell line	pH of inoculated cultures in comparison with control cultures	CPE			Titers[a]		
		Grade	Day first noted	Description of lesion	TCD_{50}	HAD_{50}	ID_{50}
BHK-21	Acid	3–4+	2	Syncytium	6.5	ND[b]	ND
Vero	Acid	2–3+	3	Mostly necrosis, some syncytia	4.5	4.5	ND
HeLa	—	2+	8	Necrosis	4.5	5.7	6.0
LLC-MK_2	—	2+	3	Necrosis	4.5	4.7	4.7
PS	—	0	—	—	0	0	1.5
MA-111	—	0	—	—	0	0	3.7

[a] Titers expressed as negative $\log_{10}$/ml; ID_{50} titers determined by subinoculation into BHK-21 cell cultures on day 7.

[b] ND, not done.

but did reduce that of vaccinia virus (Table II), Nariva may be an RNA virus.

HA by Nariva virus. A hemagglutinin was not detected in the fluids of infected Vero and BHK-21 cultures or in these fluids plus the frozen–thawed cell extract of BHK-21 cultures, and it was not until infected Vero cultures plus fluid were sonicated that a satisfactory HA preparation was obtained. This preparation was active in agglutinating guinea pig RBC but did not agglutinate 1-day chick or goose RBC suspended in saline.

TABLE II. Effect of 5-Iododeoxyuridine (IUDR) on Multiplication of Nariva Virus in BHK-21 Cultures.

Virus	Inoculum (total $\log_{10}$ TCD_{50})	Virus yield ($\log_{10}$ TCD_{50}/ml)	
		No IUDR	10^{-4} IUDR
Nariva	2.8	7.4	7.4
EE	4.5	10.2	10.5
Vaccinia	5.5	8.2	2.5

In a representative experiment on the effect of TE treatment on Nariva HA activity (Table III), an 8-fold increase in HA titer was recorded for Nariva while TE treatment of DA virus, a known myxovirus, resulted in a 32-fold increase of HA activity. Table III shows that the RBC receptors utilized by Nariva HA were not susceptible to the action of trypsin or of RDE. The effectiveness of the RDE treatment in this experiment is demonstrated by the significantly lessened HA activity of DA virus when reacted with RDE-treated RBC.

Temperature had a marked effect on HA by Nariva in reaction with guinea pig RBC; HA was greatest at 4°, 4-fold less at 23° and undetectable at 37° (Table IV). With 1-day chick RBC, no agglutination occurred at any of these temperatures.

In experiments on the adsorption and elution characteristics of Nariva HA in reaction with guinea pig RBC, 50–75% of the available HA was adsorbed in the 90-min period and the adsorbed HA was essentially quantitatively recovered in the 2-hr elution period (Table V). Since these RBC were fully agglutinable by fresh Nariva HA, it is concluded that this was not enzymatic elution

TABLE III. Nariva HA Activity as Affected by Tween–Ether (TE) Treatment and by Treatment of Guinea Pig RBC with Receptor-Destroying Enzyme (RDE) and Trypsin.

HA preparation		Treatment of guinea pig RBC		
Virus	Treatment	None	RDE	Trypsin
Nariva	No TE	64[a]	32	128
	TE	512	256	256
DA	No TE	128	8	64
	TE	4096	32	2048

[a] HA units/0.2 ml.

TABLE IV. Nariva HA Activity at Different Temperatures of Incubation.

Nariva HA preparation	Temp (°)	HA units/0.2 ml: Guinea pig RBC	HA units/0.2 ml: Chick RBC
No TE treatment	37	2	2
	23	4	2
	4	16	2
TE treated	37	4	4
	23	64	4
	4	256	4

accompanied by an alteration of the receptors. A similar conclusion was reached by a slightly different experimental approach. When the guinea pig RBC from the 37 and 23° tests shown in Table IV were resuspended and reincubated at 4°, the resultant end points were equal to that in the test originally incubated at 4°. Any of the titrations were rapidly converted to negative at this point by incubation at 37° for at least 20 min; on resuspension of the RBC and reincubation of the test at 4°, however, a positive HA titration to titer was reestablished. This procedure was repeated for at least 4 cycles without the slightest loss in HA titer.

Serological studies with Nariva virus. In HI test, Nariva HA (4 units) was not inhibited by antisera to the following viruses: parainfluenza types 1, 2, 3, and 4, Willowbrook, mumps, DA, Newcastle disease (ND), measles, rubella, and peromyscus (12), but was inhibited by its homologous antiserum to a titer of 1:320–1:640. Normal rabbit, mouse, and guinea pig sera were included as controls, and homologous HI activity of the antisera was verified in prior or simultaneous testing. A Nariva antiserum likewise failed to inhibit hemagglutination by peromyscus virus.

In CF test, Nariva antigen did not react with antisera to respiratory syncytial, influenza A, and influenza B viruses, and results were also negative in reciprocal cross-CF tests with Nariva, rabies, lymphocytic choriomeningitis, ectromelia and ND viruses.

Attempted propagation in mosquito larval cells. Evidence being lacking that Nariva fulfills the definition of an arbovirus in terms of ability to multiply in and be biologically transmitted by arthropods (13), propagation of the virus was attempted in continuous lines of *Aedes aegypti* and *A. albopictus* larval cells (14). CPE was not observed, nor was viral multiplication detected by subinoculation of culture fluid into infant mice and BHK-21 cell cultures.

Discussion. Although the negative results in mosquito larval cell cultures are suggestive, they do not answer conclusively the paramount question of whether Nariva virus multiplies in insects and thus qualifies as an arbovirus. Nonetheless, other experimental results do show that Nariva has certain biological and physical properties that are not shared by any of the well-studied arboviruses and bear some resemblance to those of myxoviruses.

The marked capacity of Nariva to induce syncytia in BHK-21 cell cultures and, to a lesser extent, in Vero cultures is a characteristic most frequently associated with the mumps–NDV–parainfluenza group of myxoviruses (15). The reported insensitivity to RDE and trypsin of RBC receptors for Nariva HA is, admittedly, shared by the receptors for hemagglutinins of certain recognized arboviruses. On the other hand, al-

TABLE V. Adsorption and Elution Characteristics of Nariva HA Interacting with 2% Guinea Pig RBC.

Reaction sequence sampling	HA units/0.2 ml of TE-treated Nariva HA: + Saline A[a]	+ Saline B[a]	+ 2% RBC A	+ 2% RBC B
0 min	128	32	128	32
Supernatant after 90-min adsorption at 4°	128	32	32	16
Cold saline wash of RBC	—	—	2	2
Supernatant after 60-min elution at 37°	128	32	64	8
Supernatant after 120-min elution at 37°	128	32	64	8

[a] A and B represent duplicate experiments.

though an active elution mechanism has been demonstrated for many of the myxoviruses, measles virus is an exception and the receptors it utilizes are not sensitive to neuraminidase or the action of influenza virus (16, 17).

Serological studies by both HI and CF tests have provided no evidence of an antigenic relation of Nariva virus to any of the better known myxoviruses or a limited number of other nonarboviruses. Studies on the morphology of this virus particle and on the morphogenesis of the virus during replication should help clarify its nature.

Summary. Nariva virus induced syncytium formation in BHK-21 and Vero cultures. Such infected cultures showed positive hemadsorption for guinea pig red blood cells (RBC). A hemagglutinin for guinea pig RBC was extracted from infected Vero cells by sonication. The hemagglutinin did not act on 1-day chick or goose RBC, and it showed an enhancement in titer upon treatment with Tween–ether. The hemagglutination reaction was highly temperature-dependent, with optimal titers being obtained at 4° and no activity manifested at 37°. Serological studies by hemagglutination-inhibition and complement-fixation indicated no antigenic relation to the better known myxoviruses or to a number of other viruses. The nucleic acid component of Nariva virus is probably RNA.

The HeLa cell line was kindly supplied by Dr. B. Mandel of New York, N. Y. The hemagglutinating preparation of DA virus was generously donated by Dr. G. D. Hsiung of Yale University and the West Haven VA Hospital. The competent technical assistance of Miss C. Mongillo is gratefully acknowledgd.

1. Tikasingh, E. S., Jonkers, A. H., Spence, L., and Aitken, T. H. G., Am. J. Trop. Med. Hyg. **15**, 235 (1966).

2. Karabatsos, N. and Buckley, S. M., Am. J. Trop. Med. Hyg. **16**, 99 (1967).

3. Liebhaber, H., Krugman, S., McGregor, D., and Giles, J. P., J. Exptl. Med. **122**, 1135 (1965).

4. Clarke, D. H. and Casals, J., Am. J. Trop. Med. Hyg. **7**, 561 (1958).

5. Norrby, E., Proc. Soc. Exptl. Biol. Med. **111**, 814 (1962).

6. Sartorelli, A. C., Fischer, D. S., and Downs, W. G., J. Immunol. **96**, 676 (1966).

7. Hsiung, G. D., "Diagnostic Virology," p. 51. Yale Univ. Press, New Haven, Connecticut (1964).

8. Karabatsos, N., J. Immunol. **91**, 76 (1963).

9. Salzman, N. P., Virology **10**, 150 (1960).

10. Flanagan, J. F. and Ginsberg, H. S., J. Exptl. Med. **116**, 141 (1962).

11. Green, M., Virology **18**, 601 (1962).

12. Morris, J. A., Bozeman, F. M., Aulisio, C. G., and Shirai, A., Proc. Soc. Exptl. Biol. Med. **113**, 296 (1963).

13. Casals, J. and Reeves, W. C., *in* "Viral and Rickettsial Infections of Man" (F. L. Horsfall, Jr. and I. Tamm, eds.), 4th ed., p. 580. Lippincott, Philadelphia, Pennsylvania (1965).

14. Singh, K. R. P., Current Sci. (India) **36**, 506 (1967).

15. Pereira, H. G., Advan. Virus Res. **8**, 245 (1961).

16. Periés, J. R. and Chany, C., Proc. Soc. Exptl. Biol. Med. **110**, 477 (1962).

17. Norrby, E., Arch. Ges. Virusforsch. **12**, 164 (1962).

Received Nov. 1, 1968. P.S.E.B.M., 1969, Vol. 130.