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Characterization of Echovirus Types 7, 12, and 29 Hemagglutinins for Human and Monkey Erythrocytes (37943)

LABIUS N. MUTANDA[1,2] AND MAURICE A. MUFSON

Departments of Medicine and of Preventive Medicine and Community Health, Abraham Lincoln School of Medicine, University of Illinois at the Medical Center, Chicago, Illinois 60680, and the West Side Veterans Administration Hospital, Chicago, Illinois 60680

Since the demonstration of hemagglutinins associated with certain human enteric viruses by Goldfield *et al.*, use has been made of this property to type hemagglutinating field strains (1). Among the enterovirus serotypes that ordinarily grow in both human and monkey cells, 14 ECHOvirus and 4 Coxsackie B virus types agglutinate human erythrocytes (2). Recently, Mutanda and Munube demonstrated that ECHOvirus types 7 and 12 agglutinated african green monkey (*Cercopithecus aethiops*) erythrocytes (3). In this report we describe the agglutination of this species of simian erythroctyes by ECHOvirus type 29. To further investigate this phenomenon, the hemagglutinins of ECHOvirus types 7, 12, and 29 for human and monkey erythrocytes were characterized.

Materials and Methods. Viruses. The field strains of viruses tested were isolated from either stool or throat swabs taken from children. Field strains of ECHOvirus types 7 (Lopes, Parker, and Patton) and 12 (White, Nodgress, and Acosta) were kindly supplied by Drs. E. H. Lennette and Nathalie Schmidt of the California State Department of Health. Each isolate was retyped as ECHOvirus 7 or 12 respectively. The other viruses used in the investigation were prototype strains obtained from the Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Immune sera. Pooled immune sera (horse), also obtained from the Reference Reagents Branch and prepared according to the method of Schmidt, Guenther, and Lennette, were used to type the isolates (4).

Tissue cultures. Primary green or rhesus monkey kidney cell monolayers were grown on Eagle's Essential Medium (Hank's Salts) supplemented with 10% calf serum and maintained on Medium 199 containing 2% inactivated (56°, 30 min) calf serum. Five-tenths percent (final concentration) of SV_5 immune serum was added to both growth and maintenance media. Human diploid fibroblast cells (strain HEL—human embryonic lung) were grown and maintained on the same media as the monkey kidney cells but without the addition of SV_5 serum. To all media were added to final concentration penicillin (100 U/ml), streptomycin (100 gamma/ml), and amphotericin B (0.001 mg/ml). Inoculated cultures were incubated at 33° on rotating drums and examined at 3-day intervals for cytopathic effects.

Typing of isolates. Isolates which agglutinated human or monkey erythrocytes were typed by serum neutralization tests. Three-tenths of each serum pool was heat inactivated at 56° for 30 min and mixed with an equal volume of the test virus diluted to contain 100 TCD_{50}. The mixtures were in-

[1] WHO Fellow from the East African Virus Research Institute, Entebbe, Uganda.

[2] Reprint requests to: L. N. Mutanda c/o Dr. M. A. Mufson, Department of Medicine, Abraham Lincoln School of Medicine, Box 6998, Chicago, IL 60680.

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cubated at 37° for 1 hr and 0.2 ml of the serum–virus mixture was inoculated into two roller tube cell cultures. As a virus control, an inoculum was used consisting of equal volumes of test virus and phosphate-buffered saline (PBS). All tests were conducted in roller tube cultures of primary green or rhesus monkey kidney cells.

Hemagglutination test. HA tests were performed in microtiter plates using 0.05 ml of twofold serial dilutions of virus in PBS at pH 7.5 and an equal volume of 1 percent erythrocyte suspensions (5). The tests were performed at 4 and 37°.

Treatment of viral hemagglutinin with chemical reagents. Viral hemagglutinins were treated with chemical reagents according to the technique of Lerner, Cherry, and Finland (6). Briefly, equal amounts (0.5 ml) of viral suspensions and each of the various reagents in 0.85% saline were mixed according to the concentrations, temperature, pH, and time specified in Table IV. The control samples were tested in parallel with the treated viral suspension under the same conditions, but with the substitution of diluent in place of the specific reagent.

The reagents used (and source of each) were: Ribonuclease (Sigma) and Receptor Destroying Enzyme (RDE) (Microbiological Associates, MD); potassium periodate (Fisher); Phospholipase C (Sigma); trypsin (Flow Laboratory); α-chymotrypsin, papain, *para*-hydroxymercuribenzoate (PMB), glutathione (GSH) and *N*-ethylmaleimide (Sigma); formaldehyde (Mallinckrodt Chemical Works); and glutaraldehyde (Polysciences Inc.). Ribonuclease, RDE, trypsin, PMB, GSH, and *N*-ethylmaleimide were prepared according to Lerner, Cherry, and Finland and the remainder of the reagents according to the formula of Castro, Nicoletti, and Cocuzza (6, 7). Formaldehyde was inactivated by the addition of sodium bicarbonate and the pH adjusted to 7.2.

Results. Hemagglutination studies. Of nine enterovirus serotypes which agglutinated human erythrocytes, only ECHOviruses types 7, 12, and 29 also agglutinated monkey erythrocytes (Table I). The HA titers of these three viruses were comparable using either human or monkey erythrocytes.

On passage of ECHOvirus types 7, 11, 12, and 29 in HEL cells, HA titers with human erythrocytes were substantially reduced for the 3 strains of type 12 and the 1 type 29 strain tested (Table II). After passage in HEL, however, the White and Norgress strains of ECHOvirus type 12 failed to agglutinate monkey erythrocytes or agglutinated these cells to very low levels.

Elution of ECHOviruses from human and monkey erythrocytes was done according to the technique of Lerner, Cherry, and Finland (6). Briefly, ECHOviruses types 7, 12, and 29 were tested for elution from human and monkey erythrocytes by mixing 4.5 ml of virus suspension with 0.5 ml of a 50% suspension of human or monkey erythrocytes and incubating the mixture at 37°. After 2, 4, and 24 hr of incubation, aliquots were centrifuged gently and the supernatant fluids from the ECHOvirus 7 and 29 tests failed to agglutinate human and monkey erythrocytes, and those from ECHOvirus 12 contained comparable low levels of HA activity for both species of erythrocytes; the titers were unchanged with increasing time of incubation.

Virus was eluted from the erythrocytes by washing 8 times with PBS (6). The human and monkey erythrocytes after elution were then resuspended in PBS to a 1% dilution and retested for HA activity against ECHOvirus types 7, 12, and 29. Human and monkey erythrocytes after prior adsorption and elution retained their receptors for the viral hemagglutinins of ECHOvirus types 12 and 29 (Table III). The decreased HA titers of ECHOvirus 7 with eluted human and monkey erythrocytes suggest that either their receptors for this virus were altered as a result of HA or that considerable virus could not be removed from them by repeated washing.

Effect of various reagents on ECHOvirus hemagglutinins. The sensitivity of the hemagglutinins of ECHOvirus types 7, 11, 12, and 29 to certain enzymes and chemicals which destroy either carbohydrates, lipids, or proteins was studied by incubating equal amounts of virus suspension with each re-

TABLE I. Enterovirus Agglutination of Human and Monkey Erythrocytes.[a]

Strain	Virus type	Species of Erythrocytes	
		Human	Monkey
SG21629	ECHO 3	256	<2
SG21634	ECHO 3	128	<2
SG20010	ECHO 6	128	<2
SG20094	ECHO 6	64	<2
SG20095	ECHO 6	32	<2
SG20126	ECHO 6	32	<2
SG238	ECHO 7	256	256
SG1428	ECHO 7	64	64
Lopes	ECHO 7	64	32
Parker	ECHO 7	64	64
Patton	ECHO 7	<2	<2
SG19882	ECHO 11	32	<2
SG20002	ECHO 11	8	<2
SG20104	ECHO 11	16	<2
103675	ECHO 11	256	<2
SG21627	ECHO 12	32	32
White	ECHO 12	512	512
Norgress	ECHO 12	512	512
Acosta	ECHO 12	32	16
SG19931	ECHO 13	32	<2
SG20069	ECHO 19	64	<2
J-V-10	ECHO 29	32	32
SG19915	Coxsackie B-5	64	<2
SG19933	Coxsackie B-5	16	<2
SG20045	Coxsackie B-5	256	<2
SG20056	Coxsackie B-5	128	<2
SG20330	Coxsackie B-5	64	<2
SG20618	Coxsackie B-5	8	<2

[a] In this and subsequent tables, the titers are expressed as the reciprocal of the final dilution of virus suspensions giving complete hemagglutination.

agent listed in Table IV. Treated ECHOvirus types 7 and 12 hemagglutinins reacted similarly with human or monkey erythrocytes. However, ECHOvirus type 29 hemagglutinin was trypsin sensitive when tested with monkey erythrocytes but not human erythrocytes. Glutaraldehyde and *para*-hydroxymercuribenzoate inhibited the hemagglutinating activity of all of the ECHOviruses tested.

Discussion. The agglutinability of monkey erythrocytes by ECHOvirus types 7, 12, and 29 is of particular practical importance in the presumptive categorization of enterovirus field strains. Since to our knowledge only ECHOvirus 7, 12, and 29 agglutinate both human and monkey erythrocytes, this property can be used to differentiate isolates of these types from other enteroviruses. Agglutination of monkey erythrocytes by these serotypes appears to be a general characteristic because the prototype and several field strains reacted in a similar manner.

Loss of the hemagglutinating activity of ECHOviruses after passage in tissue culture cell lines was observed by Maisel and coworkers and Podoplekin (8, 9). Our result with ECHOvirus types 12 and 29 agree with their findings. Further, this reduction in HA activity of ECHOvirus type 12 after tissue culture passage was especially marked for monkey erythrocytes, suggesting that the hemagglutinin of this virus for monkey erythrocytes has different properties from those for human erythrocytes. Elution tests

TABLE II. Effect of Passage in HEL Cells on Echovirus Hemagglutinins.

Strain	Virus type	Cells grown	Passage level[a]	Erythrocytes: Human	Erythrocytes: Monkey
Wallace	ECHO 7	MK	—	256	128
Wallace	ECHO 7	HEL	1	128	128
Wallace	ECHO 7	HEL	2	32	32
Parker	ECHO 7	MK	—	64	64
Parker	ECHO 7	HEL	1	64	64
Gregory	ECHO 11	MK	—	64	<2
Gregory	ECHO 11	HEL	1	32	<2
103675	ECHO 11	MK	—	256	<2
103675	ECHO 11	HEL	1	128	<2
Travis	ECHO 12	MK	—	2048	1024
Travis	ECHO 12	HEL	1	512	64
Travis	ECHO 12	HEL	2	128	64
White	ECHO 12	MK	—	512	512
White	ECHO 12	HEL	1	128	<2
White	ECHO 12	HEL	2	128	8
Acosta	ECHO 12	MK	—	256	256
Acosta	ECHO 12	HEL	1	128	32
Acosta	ECHO 12	HEL	2	64	32
Norgress	ECHO 12	MK	—	512	512
Norgress	ECHO 12	HEL	1	64	<2
Norgress	ECHO 12	HEL	2	128	4
J-V-10	ECHO 29	MK	—	32	32
J-V-10	ECHO 29	HEL	1	8	8

[a] Multiple passages in MK prior to passage in HEL cells for indicated times.

with ECHOviruses types 7, 12, and 29 and human and monkey erythrocytes failed to uncover differences in the interaction of ECHOviruses with receptors on these cells.

Chemical treatment of ECHOvirus types 7, 11, 12, and 29 provided indirect evidence that the hemagglutinins of these serotypes are proteins since all HA activity was abolished following treatment with reagents which interact with proteins. Inhibition of enterovirus hemagglutination by aldoses and a possible mechanism was investigated by Lerner and coworkers (10). They found that aldehydes were least effective in preventing HA with ECHOvirus types 7 and 12 and reovirus 2. Formaldehyde, however, was active against all of the tested viruses. On the contrary, Castro and coworkers did not detect the inhibitory effect of formaldehyde on the hemagglutinins of RV, H-1, and X14 picodnaviruses (7). Similarly, under the conditions of our tests, formaldehyde had no or little inhibitory activity on ECHOvirus hemagglutinins.

TABLE III. Agglutinability of Virus-Treated Erythrocytes.

Strain	Virus type	Normal Erythrocytes: Human	Normal Erythrocytes: Monkey	Eluted Erythrocytes: Human	Eluted Erythrocytes: Monkey
Wallace	ECHO 7	256	256	32	32
Travis	ECHO 12	64	128	64	64
J-V-10	ECHO 29	16	16	8	8

TABLE IV. Effect of Different Reagents on Hemagglutinins of Echoviruses.

Reagents	Concentration	pH	Temp.	ECHO 7 Wallace		ECHO 7 Parker		ECHO 11 Gregory		ECHO 12 Travis		ECHO 12 White		ECHO 29 JV-10	
				Human RBC	Monkey RBC	Human RBC	Monkey RBC	Human RBC	Monkey RBC	Human RBC	Monkey RBC	Human RBC	Monkey RBC	Human RBC	Monkey RBC
None	—	7.5	37C	128	128	64	64	32	<2	128	64	256	256	32	32
Ribonuclease	2 mg/ml	6.0	37C	64	64	64	64	32	<2	32	64	ND	ND	32	16
RDE	Reconstituted	6.8	37C	64	64	ND[a]	ND	32	<2	64	64	128	128	32	32
Potassium periodate	0.25%	6.2	4C	64	64	64	64	16	<2	64	32	ND	ND	16	16
Phospholipase C	0.1 U/ml	6.5	23C	64	64	64	64	32	<2	128	64	128	64	16	16
Trypsin	0.8%	7.2	37C	128	128	64	64	32	<2	128	128	256	256	32	<2
α-Chymotrypsin	98 U/ml	6.2	37C	64	64	64	32	32	<2	128	128	256	128	32	32
Papain	20 U/ml	5.4	37C	128	128	64	32	32	<2	128	128	256	256	32	32
para-hydroxymercuri-benzoate	M/40,000	7.8	37C	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Glutathione	M/200	5.0	37C	64	64	ND	ND	32	<2	64	64	ND	ND	32	16
N-Ethylmaleimide	M/20	5.7	37C	64	64	ND	ND	32	<2	32	64	64	64	16	16
Glutaraldehyde	6%	7.2	37C	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Formaldehyde	0.2%	7.0	4C	128	64	64	64	32	<2	128	128	256	128	16	16

[a] ND = Not done.

The hemagglutinin of ECHOvirus type 29 was trypsin sensitive only when tested using monkey erythrocytes. This enzyme is known to catalyze the hydrolysis of only those peptide bonds in which the carbonyl function is donated by either lysine or arginine. It appears, therefore, that ECHOvirus type 29 has separate combining sites for the receptors on human and on monkey erythrocytes. Whether or not ECHOvirus types 7 and 12 have different combining sites for the receptors on the two species of erythrocytes needs further investigation.

It is unlikely that failure of ECHOvirus 29 to agglutinate monkey erythrocytes after incubation with trypsin could have been due to residual action of enzyme on erythrocytes since all of the other tested ECHOviruses, after trypsin treatment, agglutinated both human and monkey erythrocytes. Further, if residual trypsin was only active against monkey red cells, then ECHOvirus types 7 and 12 would have failed to agglutinate erythrocytes of this species, but this was not the case.

Summary. Enterovirus field and prototype strains were tested for agglutination using human and African Green Monkey erythrocytes. Only ECHOvirus types 7, 12, and 29 agglutinated monkey erythrocytes; the titers were similar to those obtained with human erythrocytes. Passage of these ECHOviruses in human diploid fibroblast cells caused a reduction in hemagglutination titers of some of the strains. Human and monkey erythrocytes after prior adsorption and elution still retained their receptors for ECHOvirus hemagglutinins. Treatment of ECHOvirus 29 hemagglutinin with trypsin removed combining sites for receptors on monkey erythrocytes but not those on human erythrocytes. No differences in ECHOvirus types 7 and 12 hemagglutinins for human erythrocytes or monkey erythrocytes were detected by chemical treatment of these viruses.

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