Distribution of Cell Receptors and Simple Sugar Inhibitors During Encephalomyocarditis Virus-Cell Union

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Received for publication 5 December 1966

Encephalomyocarditis (EMC) virus was studied for ability to agglutinate erythrocytes of various species. Human, rat, and guinea pig erythrocytes, as well as those from young rabbits, were readily agglutinated. Cells from older rabbits absorbed virus poorly, and showed little agglutination. Uptake of virus by rabbit brain also diminished with age. Various mouse tissues absorbed virus about equally well. Hemagglutination-inhibition studies demonstrated that a number of simple sugars, particularly glucose and galactosamine, interfered with uptake of virus by cells. Dextran sulfates were highly active inhibitors of EMC hemagglutination. Attempts to localize the site of action of the sugars on virus or cell are described. Treatment of virus with periodate or *p*-chloromercurobenzoate, and acetylation of virus, inhibited hemagglutination, but acetylation of semipurified receptor did not. Clarification of the nature of the virus-cell union will require studies to identify possible specific sugars in the virus capsid and the cell receptor.

Attachment of enteroviruses to a surface receptor site has been clearly shown by many investigators to be a specific, nonenzymatic reaction essential for establishment of infection. McLaren, Holland, and Syverton (26) demonstrated that the unique species susceptibility of primate cells to poliomyelitis and related viruses was dependent upon specific membrane receptors. Further investigations by Holland (17), Kunin (19, 20), and others (1, 10, 12, 16, 21, 29) have variously attempted to relate the relative abundance of enterovirus-specific receptors to organ and tissue tropisms, age-specific susceptibility, virus virulence, derivation of virus-sensitive cells in tissue culture, and selective virus sensitivity of neoplastic tissues. The enterovirus receptors appear to consist of multiple lipoproteins which differ in sensitivity to selective enzyme destruction and to treatment with periodate (22, 27) and anticellular serum (14, 28, 31). Mutual interference between viruses which attach to similar sites has been demonstrated (8, 9). Differences between susceptibility of monkey and human cells to rhinovirus strains has been attributed to the presence or absence of specific cellular receptors (15).

The present study was undertaken with encephalomyocarditis virus to characterize the importance of virus-cell union in the pathogenesis

of infection with this agent. This virus was of special interest because of its close physical and chemical resemblance to human enteroviruses, its broader host range, stability, and its ability to agglutinate erythrocytes. It also appeared particularly suitable to use this virus to extend the recent observations by Lerner and co-workers (24) on the blocking of hemagglutination by reoviruses and human enteroviruses by certain simple sugars.

MATERIALS AND METHODS

Virus and tissue culture. Encephalomyocarditis virus (EMC) with a history of 80 mouse passages and 7 further passes through mouse embryo tissue culture was obtained from John Craighead, Department of Pathology, Harvard Medical School (5). The virus was passed four more times in secondary cultures of mouse embryo cells, prepared by trypsinization overnight at 4 C, of minced 1- to 2-cm mouse embryos and cultivation in medium 199 containing 10% calf serum with penicillin and streptomycin added. Fully grown monolayers in 32-oz prescription bottles were harvested in ethylenediaminetetraacetate (EDTA) and subcultured. Second-passage cells were permitted to form monolayers, washed with Hank's balanced salt solution (BSS), and infected in 2% calf serum-medium 199 with crude virus. Cells and fluid were harvested at 24 hr when maximal cytopathogenic effect was noted. Virus was then semipurified by a method described by Faulkner et al., (11). Harvests were

pooled, frozen and thawed three times, and centrifuged at $10,000 \times g$ for 10 min at 0 C. The supernatant fluids were centrifuged in a Spinco ultracentrifuge (rotor 50) for 2 hr at 50,000 rev/min. Sediments were taken up in 0.02 M phosphate buffer (pH 7.4) containing 2 to 3 mg of crystalline trypsin, incubated for 1 hr at 37 C, and added to a diethylaminoethyl (DEAE) cellulose column (1 by 25 cm) previously equilibrated with the same buffer. Virus was eluted in 5-ml volumes from the column by a gradient established through a 250-ml mixing flask containing 0.02 M phosphate buffer and a reservoir containing 0.5 M phosphate buffer (pH 7.4). Virus appeared in two peaks, an early one after 35 to 45 ml of effluent, and a later, higher-titered peak at 100 to 150 ml. Virus obtained from the second peak was dialyzed overnight against 1,000 ml of borate buffer at 4 C, and was stored for use in hemagglutination tests. Virus prepared by this method had hemagglutinin titers of 1:132 to 1:2,560. In other studies, more highly purified virus, prepared by two passages through calcium phosphate columns prepared by the method of Faulkner et al. (11), was used; this virus appeared in a single peak by use of the elution procedure described above.

Radiolabeled EMC virus. Carbon-14 labeled virus was prepared by infecting 10 prescription bottles (32 oz) containing primary mouse embryo cells with 1 ml of crude EMC virus. Virus-cell mixtures were incubated for 1 hr at 37 C, and unabsorbed virus was removed by two washings with BSS. A 200-ml amount of BSS containing 0.5 mc of ^{14}C -amino acid mixture (specific activity, 0.5 mc per 0.355 mg; New England Nuclear Corp., Boston, Mass.), plus 2 ml of $100 \times$ vitamins (Microbiological Associates, Inc., Bethesda, Md.) and penicillin and streptomycin was prepared. The pH was adjusted to 7.4 with 1 N NaOH, and the medium was dispensed into the 10 bottles. Virus destroyed the cell sheet within 24 hr; fluids were harvested and treated by chromatography on a DEAE cellulose column as described above. Peaks of radioactivity coincided with those of virus; only the second peak was used in the experiments to be described. Pools of ^{14}C -labeled virus, after dialysis against borate buffer, contained 700 to 800 counts per min per 0.1 ml and hemagglutinin titers with human O erythrocytes of 1:160 to 1:320. Radioactivity was measured by adding 0.1 ml of fluid to 10 ml of scintillator and counting in a Packard refrigerated scintillation counter. All samples were tested in duplicate.

Hemagglutination. Erythrocytes were collected in a heparinized syringe, and washed three times with borate buffer (0.05 H_3BO_3 in 0.12 M KCl, pH 8.0) as described by Craighead and Shelakov (7). Virus-cell mixtures, unless otherwise stated, consisted of 0.5 ml of virus dilution added to 0.5 ml of an 0.4% suspension of erythrocytes. Mixtures were incubated for 2 hr at 4 C in multiple-cup plastic trays (Limbro Manufacturing Co., New Haven, Conn.), and read by pattern. Human O erythrocytes, drawn freshly each day from a single donor, were used in all virus-inhibition experiments.

Sugar inhibition tests. Solutions of each sugar, dextrans, and dextran sulfates were prepared in borate buffer, and the pH was adjusted to 8.0 when required

with 1 N KOH. In sugar-virus mixtures, 0.25 ml of sugar solution was added to 0.25 ml of EMC virus which had been purified by two passages through calcium phosphate columns. Two and four hemagglutinating units of virus were used in most experiments. Incubation was conducted at 4 or 37 C for 2 hr; 0.5 ml of 0.4% washed human erythrocytes in borate buffer was then added, and incubation was continued at the same temperature for 4 hr more. End points were read by pattern. In sugar-cell mixtures, 0.25 ml of sugar solution was added to 0.5 ml of erythrocytes. Incubation was conducted as described above; then 0.25 ml of virus was added prior to the second stage of incubation.

Dextrans and dextran sulfates of various molecular size were obtained from Sigma Chemical Co., St. Louis, Mo.

Periodate oxidation. EMC virus, semipurified by one passage through a calcium phosphate column, was dialyzed overnight with 0.05 M sodium acetate buffer (pH 4.5). A 2-ml sample of virus was treated with 50 μmoles (in 0.5 ml) of sodium periodate for 1 hr at 37 C, followed by addition of 100 μmoles of glycerol (in 10 ml) to stop the reaction. A control tube containing the same amount of virus received a mixture of periodate and glycerol which had been preincubated for 1 hr at 37 C; another control tube contained virus plus 1.5 ml of water. Each mixture was dialyzed overnight against borate buffer. (I am indebted to Wesley Volk for aid in performance of these studies and those described in the following section.)

Acetylation of virus. EMC virus, semipurified by one passage through a calcium phosphate column, was dialyzed overnight with saline-phosphate buffer (pH 7.2). Solid NaHCO_3 (100 mg) was added to three tubes containing 2-ml samples of virus. One tube was treated with 10 μliters of acetic anhydride, another, with the same volume of glacial acetic acid, and a third, with nothing more. A fourth tube received virus alone. Mixtures were incubated for 30 min at 37 C and then dialyzed overnight against borate buffer.

Acetylation of brain CaCl_2 -butanol extracts. A 2-ml amount of undiluted monkey or mouse brain, to which solid NaHCO_3 was added, were treated with acetic anhydride with five increments of 0.1 ml, at 10-min intervals. The pH was not allowed to fall below 7.0 by addition of more NaHCO_3 . An equal volume of borate buffer was added to parallel tubes of brain extract. Mixtures were dialyzed overnight at 4 C against borate buffer (followed by volume adjustment of the borate control because of uptake of fluid by the NaHCO_3 -acetic anhydride-treated extract) and tested for ability to block hemagglutination or uptake of ^{14}C -labeled EMC virus by erythrocytes as described above. In an additional experiment, monkey brain extract was concentrated twofold with Carbowax, and 5 ml was treated with a total of 0.5 ml of acetic anhydride (with control of pH).

Sulfhydryl binding. EMC virus, prepared as described above for acetylation, was treated with a final concentration of 10^{-4} M *p*-chloromercuribenzoate (PCMB) for 1 hr at 37 C (1.8 ml of virus plus 0.2 ml of 10^{-3} M PCMB), and then was dialyzed overnight

against borate buffer (pH 8.0). The control consisted for 1.8 ml of virus and 0.2 ml of water dialyzed in the same manner.

PCMB (Sigman Chemical Co., St. Louis, Mo.) was prepared by the method of Philipson and Choppin (3).

Preparation of CaCl_2 -butanol extracts of brain. Animals were killed by breaking the neck or by exsanguination. Brains were studied immediately or after storage at -70°C , and were prepared as 10% homogenates in saline or borate buffer by use of Teflon grinders. Homogenates were centrifuged at low speed and the supernatant fluid was discarded. CaCl_2 -butanol extracts were prepared by a modification of the method of Philipson et al. (27) A 5-ml amount of 2.5 M CaCl_2 , adjusted to pH 6.7 with 1 N NaOH, plus an equal volume of butanol, was added to each gram of sediment. The mixture was homogenized for 2 to 3 min and centrifuged at 4°C at $10,000 \times g$ for 1 hr. Two clear layers divided by a third solid layer were noted. The lowest (aqueous layer) was removed and recentrifuged as before. The aqueous layer was dialyzed for 18 hr at 4°C against 500 ml of 0.1 M tris (hydroxymethyl)-aminomethane (Tris) buffer (pH 7.5) containing 10^{-4} M EDTA and 10^{-3} M MgCl_2 . The Tris buffer was then removed, and the preparation was further dialyzed for 4 hr against borate buffer. Hemagglutination blocking studies were performed by incubating 0.25 ml of EMC virus and 0.25 ml of the opalescent extract for 1 hr at 37°C , and then adding 0.5 ml of a suspension of group O human erythrocytes, at 4°C . Radioactive blocking tests were performed by incubating 0.1 ml of ^{14}C -labeled EMC virus with 0.1 ml of the extract for 1 hr at various temperatures, and then adding 0.1 ml of a 1:16 dilution of human group O erythrocytes for 30 min at 37°C . Mixtures were centrifuged, and radioactivity remaining in supernatant fluids was measured in a scintillation counter.

RESULTS

EMC agglutination of erythrocytes from various species. The ability of EMC to agglutinate erythrocytes of different species is shown in Table 1. Hemagglutination was maximal at 4°C . Titers were two- to fourfold lower at 37°C with human and guinea pig cells; no agglutination was demonstrable at this temperature with rabbit cells. Human and rat cells were equally sensitive regardless of age. Newborn and adult New Zealand rabbit (The Arrows Rabbit Farm, Charlottesville, Va.) cells, however, gave divergent results. Erythrocytes from newborn rabbits were readily agglutinated and gave high hemagglutination titers, whereas adult cells were only partially agglutinated (incomplete pattern of spread-out cells) and gave lower titers. By 30 days of age or more, little or no agglutination was observed. This age-related phenomenon, observed only with rabbit cells, is shown in Fig. 1.

Uptake of EMC ^{14}C by erythrocytes. Removal of ^{14}C -labeled EMC virus from the supernatant fluids of mixtures of virus by serial dilutions of

TABLE 1. Ability of EMC virus to agglutinate erythrocytes of various species at 4°C

Species	Hemagglutination ^a	Note
Adult "O"		
Cord	+	No difference in titer with age
Adult	+	
Rabbit		Incomplete agglutination pattern with adult cells
Newborn	+	
Adult	±	
Rat		No difference in titer with age
Newborn	+	
Adult	+	
Guinea pig		
Adult	+	
Sheep		
Adult	+	
Chicken		
Newborn	—	
Adult	—	
Cat		
Adult	—	
Goat		
Adult	—	
Pig		
Adult	—	Autoagglutination at 4°C ^b
Calf	—	

^a Symbols: + = good agglutination; ± = incomplete agglutination; — = no agglutination.

^b No activity at 20 or 37°C .

FIG. 1. EMC hemagglutination titers achieved with rabbit cells obtained from animals of various ages.

human erythrocytes is shown in the upper portion of Fig. 2. A 1:16 dilution of human erythrocytes was repeatedly found to remove 50 to 60% of virus, and was used as the standard test

dilution of cells in all other studies. Most of the virus uptake occurred within the first 15 min of incubation and was independent of the temperature of incubation (Fig. 3). Standard incubation with erythrocytes in succeeding tests was conducted for 30 min at 37 C.

Uptake of ^{14}C -labeled EMC virus by rabbit erythrocytes, according to age and concentration of cells, was tested to determine whether the poor hemagglutinating property of adult rabbit cells was due to poor virus uptake or some other factor. As shown in Fig. 2, newborn rabbit cells were more active than similar dilutions of adult cells, but both removed less virus than did human erythrocytes. It thus appears that poor agglutination of adult rabbit erythrocytes by EMC virus is associated with a markedly diminished ability of these cells to take up the virus.

Carbon-14 labeled EMC virus used in these studies was shown to be specifically inhibited by high-titered rabbit antiserum (kindly provided by

FIG. 2. Removal of ^{14}C -labeled EMC virus from supernatant fluids of mixtures with serial dilutions of erythrocytes from adult human and newborn adult New Zealand rabbits. Incubation was for 1 hr at 37 C. Similar results were obtained at 0 and 20 C.

FIG. 3. Effect of incubation time and temperature on removal of ^{14}C -labeled EMC virus from supernatant fluids of mixtures with 1:16 human O erythrocytes. Control tubes contained virus in borate buffer.

Samuel Baron of the National Institutes of Health, Bethesda, Md.). Immune serum had a hemagglutination-inhibition titer of 1:2,560; pre-immunization serum titer was less than 1:10. Serial dilutions of serum were incubated with ^{14}C -labeled EMC at 37 C. Buffer controls and undiluted or serially diluted preimmunization serum permitted 50% removal of radioactivity by the cells. Dilution of the immune serum to 1:1,024 or greater completely inhibited removal of ^{14}C -labeled virus from the supernatant fluid.

Absorption of EMC virus by tissue extracts. Ten per cent homogenates of adult mouse tissues were prepared in borate buffer. These were serially diluted in buffer, and 0.5 ml of each dilution was incubated for 1 hr at 37 C with 4 hemagglutinating units of EMC virus. Mixtures were then centrifuged at low speed and supernatant fluids were tested for residual virus. Mouse brain, lung, kidney, heart, muscle, and gut diluted 1:128 to 1:256 removed all detectable hemagglutinin. No significant differences were observed among various tissues in ability to remove virus. Similar studies with homogenates of rabbit brain demonstrated a clear effect according to age. With the use of 8 hemagglutinating units of virus, the dilutions of brain capable of removing detectable virus from the supernatant fluids were as follows: newborns, 1:8; 5 days, 1:4; 11 days, 1:4; 22 days, 1:4; 37 and 40 days, 1:2; 57 days to 2 years, no absorption at dilutions of brain of 1:2 or greater.

Activity of CaCl_2 -butanol brain extracts against EMC virus. Preincubation of EMC virus with undiluted extracts of adult mouse or rhesus monkey brain reduced the EMC hemagglutinin titer from 1:320 to $<1:10$. Activity was greatly diminished by dilution of the extract and was no longer detectable at 1:8. Rabbit brain extracts appeared to be much less active, in that undiluted material reduced the titer to only 1:20 to 1:40, with no clear relation to age (newborn to 2 years), and activity was virtually absent when extracts were diluted to 1:4. These extracts were also shown to block uptake of ^{14}C -labeled EMC virus by human erythrocytes in the order: monkey more active than mouse more active than rabbit brain.

Inhibition of hemagglutination by sugars. A series of simple sugars were tested for their ability to block hemagglutination of human erythrocytes by EMC virus. Results of studies conducted at 4 and 37 C with mixtures of virus and sugars preincubated prior to addition of cells are shown in Table 2. Glucose was more effective at 4 than at 37 C. Galactosamine was active at both temperatures, but more so at 37 C. A marked "zone phenomenon" was observed with galactosamine. Very high (500 μmoles) and very low concentrations (1.6 μmoles)

TABLE 2. *Minimal inhibiting concentration of various simple sugars required to block agglutination of human erythrocytes by EMC virus^a*

Temp	Sugar	Hemagglutination units of virus			
		2	4	8	16
C 4	Glucose	50 ^b	100	>100	>100
	Galactosamine	500	500	—	—
	Rhamnose	500	>500	—	—
37	Glucose	500	500	—	—
	Galactosamine	1.6 ^c	1.6	1.6	>100
	Glucoseamine	500	500	—	—
	Fructose	25	25	>100	>100
	D-Xylose	50	100	>100	>100
	D-Arabinose	100	100	100	100
	Galacturonic acid	25	50	100	100

^a Virus-sugar mixtures were preincubated for 0.5 hr prior to addition of cells. No hemagglutination-inhibition was observed with mannose, L-arabinose, L-rhamnose, D-galactose, fucose, sucrose, *N*-acetylglucosamine, *N*-acetylgalactosamine, methyl- β -D-glucopyranoside, or α -methyl-D-glucoside tested at 500 or less μ moles per ml.

^b Minimal inhibitory concentration in micromoles per milliliter of initial concentration; final concentration after addition of cells is one-fourth of that shown.

^c Zone phenomenon noted, e.g., block at 500, no block at 100, 50, or 25, but block at 12.5 to 1.6 μ moles per ml.

of galactosamine completely blocked agglutination, but intermediate amounts of 25 to 100 μ moles were ineffective. Glucosamine was much less active than galactosamine; no blockade could be demonstrated with the *N*-acetylated derivatives of either amino sugar.

The same sugars, tested at 500 μ moles, were incubated with erythrocytes prior to addition of virus; only glucose, galactosamine, and glucosamine were able to block agglutination (Table 3). Glucose was equally active at 4 and 37 C; the other sugars were most active at 37 C.

Effect of sugars on uptake of ¹⁴C-labeled EMC virus by human erythrocytes. Sugars were tested for ability to interfere with the uptake of EMC virus by human erythrocytes to ascertain whether the ability of certain of them to block hemagglutination was due to interference with virus uptake by cells, or to other factors. For the test system, 0.1 ml of each sugar (500 μ moles per ml) was mixed with either 0.1 ml of ¹⁴C-virus or 0.1 ml of a 1:16 dilution in borate buffer of packed human erythrocytes for 2 hr at 37 C. The missing third component of either cells or virus was then added and incubation was continued for 30 min more. Supernatant fluids were tested for residual radioactivity in comparison with a control sample of virus diluted in the same final volume of borate buffer. All experiments were performed in duplicate.

Incubation of virus with cells in buffer resulted

TABLE 3. *Inhibition of agglutination of human erythrocytes by preincubation with 500 μ moles of sugars prior to addition of EMC virus^a*

Sugar	4 C		37 C	
	2 HA units	4 HA units	2 HA units	4 HA units
Glucose.....	+	+	+	+
Galactosamine.....	+	—	+	+
Glucosamine.....	—	—	+	±

^a Symbols: + = complete inhibition; ± = partial inhibition; — = no inhibition.

in removal of more than 50% of radioactivity, and similar results were obtained with sugars other than glucose (Fig. 4). Prior addition of glucose to either cells or virus almost completely blocked virus uptake by the cells. Preincubation of glucose with cells was somewhat more effective than preincubation with virus, but the observed difference was small.

Effect of dextrans and dextran sulfates on EMC hemagglutination. The blocking tests, described above for simple sugars, were extended to dextrans and dextran sulfates because of the well-known effect of these compounds on plaque size of EMC, poliomyelitis, and other viruses, and because of their chemical relation to glucose. The polymers were dissolved in borate buffer

according to the calculated weight of their simplest component, e.g., dextrans, 18 mg/ml (equivalent to 0.1 M glucose) and dextran sulfates, 27.9 mg/ml (approximately equivalent to 0.1 M glucose sulfate).

All dextran sulfates tested (varying in molecular weight from 60,000 to 40 million) were active inhibitors of hemagglutination when preincubated with 2 or 4 hemagglutinating units of virus. The solutions retained activity even when diluted as much as 1:64,000 to 1:512,000.

Dextrans were much less active than dextran sulfates, in that activity was lost when dextrans were diluted more than eightfold. The lowest molecular weight polymers (15,000 to 75,000) were most active. This is shown in Table 4 in which samples of virus with a titer of 128 were incubated with various dilutions of dextrans and tested for activity against human O erythrocytes.

Chemical treatment of EMC virus. Effect of treatment of semipurified EMC virus with periodate, acetic anhydride, and PCMB is shown in Table 5. Each reagent was effective in reducing hemagglutination activity. Sulfhydryl binding, as studied with PCMB, was less effective than periodate oxidation or acetylation. Control tubes demonstrate the specificity of each reaction.

Effect of acetylation of monkey brain extract on ability to block virus uptake by erythrocytes. The marked ability of low concentrations of galactosamine to block EMC hemagglutination, and the demonstration that acetylation of the virus under aqueous conditions destroyed hemagglutinating activity (possibly by masking amino groups) led to a study of the effect of acetylation on semipurified receptor substance. Undiluted, acetylated mouse and monkey brain extract re-

TABLE 4. *Effect of dextrans (18 mg/ml) on hemagglutination of human O erythrocytes by EMC virus^a*

Molecular wt	Dextran dilution		
	1:2	1:4	1:8
15,000-20,000	Undiluted	Undiluted	2
75,000	Undiluted	2	4
193,000	16	64	64
500,000	16	32	32
5-40 million	128	128	128
Glucose	8	16	32
Buffer control	128	128	128

^a Virus and dextrans preincubated together for 1 hr at 37 C prior to addition of cells. Values shown are the reciprocal of the maximal dilution of virus producing hemagglutination in combination with dextrans, glucose, or borate buffer.

tained ability to block agglutination of human erythrocytes by EMC virus. Virus titer was reduced from 1:160 to <1:10 by treated extracts from either animal. Borate buffer-treated controls exhibited the same or slightly lower activity (Table 6).

DISCUSSION

The present study of EMC virus-cell union may be considered as an extension of a series of studies by many groups on the nature and biological significance of specific enterovirus cellular receptors. The strain of virus employed in the present study had been highly adapted to mouse tissue, and is known to produce lesions in the brain and heart (6). Despite this gross tissue tropism, the relative abundance of receptorlike activity appeared to be about the same in all adult animal tissues studied. The ability of tissues other than heart or brain to synthesize virus must be known, however, before the significance of distribution of receptor-like substance can be properly interpreted, since the lesions may be due to reaction to the virus and may not directly reflect synthesis.

Craighead (5) reported that this strain of virus produces both large and small plaques in roughly equal proportions. The marked sensitivity of virus hemagglutination to dextran sulfate preparation strongly suggests that R+ (inhibitor sensitive) variants of EMC virus accounted for most of the hemagglutination observed in the test system used. Hemagglutination-inhibition appears to be a very sensitive method for measuring this effect. The wide variety of viruses inhibited by dextran sulfates argues against this type of reaction as accounting for the specificity of virus-cell union (2, 4, 25, 30).

FIG. 4. *Effect of incubation of EMC virus or cells with various sugars on uptake of carbon-14 labeled virus by human erythrocytes. Results are shown for virus remaining in supernatant fluid 30 min after addition of final component to the system. Similar results to D-xylose obtained with D-arabinose and galacturonic acid.*

TABLE 5. *Effect of exposure of EMC virus to various agents on ability to agglutinate human erythrocytes*

Treatment	Reagent	HA titer	Final pH
Periodate oxidation	Water	1:320	4.5 ^a
	Periodate-glycerol	1:160	4.5
	Periodate	<1:10	4.5
Acetylation	Water	1:320	—
	NaHCO ₃	1:320	8.0
	NaHCO ₃ plus acetic acid	1:320	6.8
	NaHCO ₃ plus acetic anhydride	<1:10	6.8
Sulfhydryl binding	Water	1:320	7.2 ^b
	PCMB (10 ⁻⁴ M)	1:40	7.2

^a Acetate buffer.^b Phosphate buffer.TABLE 6. *Effect of acetylation of CaCl₂-butanol extracts of rhesus monkey brain on ability to block hemmagglutination by EMC virus*

Sample	Dilution of virus						
	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Extract, untreated							
Undiluted	+	0	0	0	0	0	0
1:2	+	0	0	0	0	0	0
1:4	+	+	+	0	0	0	0
1:8	+	+	+	+	±	0	0
1:16	+	+	+	+	±	0	0
Extract, acetylated							
Undiluted	0	0	0	0	0	0	0
1:2	0	0	0	0	0	0	0
1:4	+	0	0	0	0	0	0
1:8	+	+	+	+	0	0	0
1:16	+	+	+	+	+	0	0
Buffer control	+	+	+	+	+	±	0

^a Symbols: ± = partial agglutination; + = agglutination; 0 = no agglutination.

Erythrocytes obtained from various mammalian species differed in ability to be agglutinated by EMC virus. Rabbit erythrocytes were remarkable in age-specific sensitivity to the virus. The use of radio-labeled virus demonstrated that cells from older rabbits poorly absorbed virus. Definite, but less marked age differences in ability of rabbit brain to absorb virus could also be shown. These findings are similar to a previous observation that absorption of coxsackievirus B1 by mouse brain diminished with age of the animal (19). Coxsackieviruses are well known to be markedly virulent for newborn animals. Effect of animal age on virus virulence remains to be demonstrated in rabbits with the strain of EMC virus used in the current studies.

The chemical nature of the enterovirus cellular

receptor and its complementary site on the virus must be characterized before understanding of the variable species-, tissue-, and age-specific properties of these agents will be complete. Chemotherapy, directed at this initial phase of virus infection holds great promise, since it would not require a blockade of cellular synthetic processes.

Choppin and Philipson (3) have shown that human enteroviruses treated with sulfhydryl binding agents are incapable of attaching to cellular receptors. EMC virus was also inactivated by the same type of reagent, and by periodate treatment and acetylation as well. Philipson et al. (27) reported that purified human erythrocyte receptor was inactivated by chymotrypsin, papain, venom phosphodiesterase, periodate, and sonic treat-

ment, indicating that the complex glycolipid-protein required integrity of each component for activity. In the present study, tissue receptors were not affected by acetylation. Chemical characterization of the sugar moieties according to type of virus and host receptor may be an important lead to the structure of the site of virus-cell union, in view of the observations of Gelb and Lerner (13) and Lerner et al. (24) that certain aldoses and acetylated amino sugars specifically block hemagglutination of reoviruses and other enteroviruses, and in view of the studies reported here of the effect, particularly of glucose and galactosamine, on EMC virus hemagglutination.

It is possible that complementary groups both on virus and receptor contain a sugar moiety, since it can be shown that periodate oxidation will inhibit both (23, 27). Blocking experiments in which virus or cells were preincubated with sugars prior to addition of the last component of the system do not establish which component combines with the sugar, since the blocking reaction appears to occur rapidly and about equally well regardless of timing of addition of various components to the system. The current results with various treatments of virus and semipurified receptor indicate that sulfhydryl, periodate-sensitive, and amino groups must be intact in the virus for hemagglutination, whereas groups are not essential in the receptor. Thus, if galactosamine, for example, truly plays an active part in virus cell union, it should be found in the virus capsid. Furthermore, terminal amino sugars in red cell membranes have been described only in the acetylated form (18). Attempts to demonstrate the presence of various sugars in EMC virus are underway in this laboratory.

The present studies serve to confirm the observations of Lerner and his colleagues that simple sugars appear to have an important role in enterovirus hemagglutination and the observations of Philipson et al. (27) that receptor substance can be solubilized. The exact mode by which the sugars act in inhibiting virus hemagglutination is not entirely clear, but the current data, taken together with the observations of others, indicate that a search for sugars in the virus capsid may be of value.

ACKNOWLEDGMENTS

I thank Louise Moore and Ann Bugg for able technical assistance and to A. Martin Lerner for stimulation to conduct these studies.

This investigation was supported by Public Health Service grants CC-00091 and 1-K3-AI-7534 and by the Office of the Surgeon General, U.S. Army, under the sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board.

LITERATURE CITED

1. BARON, S., R. M. FRIEDMAN, AND C. F. BUCKLER. 1963. Properties of poliovirus inhibitor from monkey brain. *Proc. Soc. Exptl. Biol. Med.* **113**:107-110.
2. BENGTSSON, S. 1965. Mechanism of dextran sulfate inhibition of attenuated poliovirus. *Proc. Soc. Exptl. Biol. Med.* **118**:47-53.
3. CHOPPIN, P. W., AND L. PHILIPSON. 1961. The inactivation of enterovirus infectivity by the sulfhydryl reagent *p*-chloromercuribenzoate. *J. Exptl. Med.* **113**:713-734.
4. COLTER, J. S., M. A. DAVIES, AND J. B. CAMPBELL. 1964. Studies of three variants of mengo encephalomyelitis virus. II. Inhibition of interaction with L cells and agar inhibitor and by protamine. *Virology* **24**:578-585.
5. CRAIGHEAD, J. E. 1965. Some properties of the encephalomyocarditis, Columbia SK and mengo viruses. *Proc. Soc. Exptl. Biol. Med.* **119**:408-412.
6. CRAIGHEAD, J. E. 1966. Pathogenicity of the M and E variants of the encephalomyocarditis (EMC) virus. I. Myocardiotropic and neurotropic properties. *J. Pathol.* **48**:333-345.
7. CRAIGHEAD, J. E., AND A. SHELOKOV. 1961. Encephalomyocarditis virus hemagglutination-inhibition test using atigens prepared in HeLa cell cultures. *Proc. Soc. Exptl. Biol. Med.* **108**:823-826.
8. CROWELL, R. L. 1963. Specific viral interference in HeLa cell cultures chronically infected with Coxsackie B5 virus. *J. Bacteriol.* **86**:517-526.
9. CROWELL, R. L. 1966. Specific cell-surface alteration by enteroviruses as reflected by viral-attachment interference. *J. Bacteriol.* **91**:198-204.
10. EGGERS, H. J., AND A. B. SABIN. 1959. Factors determining pathogenicity of variants of ECHO 9 virus for newborn mice. *J. Exptl. Med.* **110**:951-967.
11. FAULKNER, P., E. M. MARTIN, S. SVED, R. C. VALENTINE, AND T. S. WORK. 1961. Studies on protein and nucleic acid metabolism in virus-infected mammalian cells. II. The isolation, crystallization and chemical characterization of mouse encephalomyocarditis virus. *Biochem. J.* **80**:597-605.
12. FRANCIS, T., JR., AND L. W. CHU. 1953. The interaction *in vitro* between poliomyelitis virus and nervous tissue. *Intern. Congr. Microbiol.*, 6th, Rome **3**:162-170.
13. GELB, L. D., AND A. M. LERNER. 1965. Reovirus hemagglutination: inhibition by N-acetyl-D-glucosamine. *Science* **147**:404-405.
14. HABEL, K., J. W. HORNIBROOK, N. C. GREGG, R. J. SILVERBERG, AND K. K. TAKEMOTO. 1958. The effect of anticellular sera on virus multiplication in tissue culture. *Virology* **5**:7-29.
15. HAFF, R. F., B. WOHLSEN, E. E. FORCE, AND R. C. STEWART. 1966. Growth characteristics of two rhinovirus strains in WI-26 and monkey kidney cells. *J. Bacteriol.* **91**:2339-2342.

16. HARTER, D. H., AND P. W. CHOPPIN. 1965. Absorption of attenuated and neurovirulent poliovirus strains to central nervous system tissues of primates. *J. Immunol.* **95**:730-736.
17. HOLLAND, J. J. 1961. Receptor affinities as major determinants of enterovirus tissue tropisms in humans. *Virology* **15**:312-326.
18. KABAT, E. A. 1966. The nature of the antigenic determinant. *J. Immunol.* **97**:1-11.
19. KUNIN, C. M. 1962. Virus-tissue union and the pathogenesis of enterovirus infections. *J. Immunol.* **88**:556-569.
20. KUNIN, C. M. 1964. Cellular susceptibility to enteroviruses. *Bacteriol. Rev.* **28**:382-390.
21. KUNIN, C. M., AND W. S. JORDAN, JR. 1961. In vitro adsorption of poliovirus by noncultured tissues, effect of species, age and malignancy. *Am. J. Hyg.* **73**:245-257.
22. LERNER, A. M., E. J. BAILEY, AND J. R. TILLOTSON. 1966. Enterovirus hemagglutination: inhibition by several enzymes and sugars. *J. Immunol.* **95**:1111-1115.
23. LERNER, A. M., J. D. CHERRY, AND M. FINLAND. 1963. Hemagglutination with reoviruses. *Virology* **19**:58-65.
24. LERNER, A. M., L. D. GELB, J. R. TILLOTSON, M. M. CARRUTHERS, AND E. J. BAILEY. 1966. Enterovirus hemagglutination: inhibition by aldoses and a possible mechanism. *J. Immunol.* **96**:629-636.
25. LIEBHABER, H., AND K. K. TAKEMOTO. 1963. The basis for the size differences in plaques produced by variants of encephalomyocarditis (EMC) virus. *Virology* **20**:559-566.
26. McLAREN, L. C., J. J. HOLLAND, AND J. T. SYVERTON. 1959. The mammalian cell-virus relationship. I. Attachment of poliovirus to cultivated cells of primate and nonprimate origin. *J. Exptl. Med.* **109**:475.
27. PHILIPSON, L., S. BENGTSSON, S. BRISHAMMAR, L. SVENNERHOLM, AND O. ZETTERQVIST. 1964. Purification and chemical analysis of the erythrocyte receptor for hemagglutinating enteroviruses. *Virology* **22**:580-590.
28. QUERSIN-THIRY, L. 1958. Action of anticellular sera on virus infection. I. Influence on homologous tissue cultures infected with various viruses. *J. Immunol.* **81**:253-260.
29. QUERSIN-THIRY, L. 1961. Interaction between cellular extracts and animal viruses. I. Kinetic studies and some notes on specificity of the interaction. *Acta Virol.* **5**:141-152.
30. ROUHANDEH, H., R. R. CHRONISTER, AND M. L. BRINKMAN. 1965. Inhibition of poliovirus minute-plaque mutants and echoviruses by sulfated polysaccharides. *Proc. Soc. Exptl. Biol. Med.* **118**:1118-1124.
31. TIMBURY, M. C. 1963. Inhibition of viral replication by anticellular serum. *Virology* **20**:629-635.