Hemagglutinins Associated with Certain Human Enteric Viruses.* (23609)

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The association of hemagglutinins (HA) with viruses has increased continuously since the classical work with influenza virus(1,2) so that now, in addition to the myxoviruses, HA are associated with the pox viruses, the arbor viruses and, recently, with certain simian viruses recoverable from monkey kidney cell cultures(3). Study of the HA phenomenon in the case of the myxoviruses has led to new concepts of virus-cell relations and in all cases the phenomenon has had immediate or potential practical value as the basis for an *in vitro* method of viral antigen assay or of serologic work. Hence, the new demonstration of HA in relation to a virus group is of possible interest from both the fundamental and the practical standpoints.

This communication will describe in preliminary fashion hemagglutinating properties discovered in relation to several members of the ECHO and Coxsackie groups of viruses. This discovery resulted from efforts to investigate the possibility that certain enteric viruses possessed the ability to agglutinate trypsin-treated red cells similar to that recently reported for the newly isolated respiratory virus, 2060(4); in the course of these efforts it was observed that untreated human erythrocytes of Group O, included for control purposes, were agglutinated by a number of the viral agents tested.

Methods. Hemagglutination. Blood was collected from Group O donors in acid-citrate-dextrose and stored in the refrigerator for no more than a week prior to use. Red cells were washed 3 times with veronal-saline buffer set at pH 7.3 and resuspended finally in 1% concentration. Hemagglutinin (HA) titrations were performed in Kahn tubes by the addition of 0.1 ml of cell suspension to 0.4 ml of serial 2-fold dilutions of virus preparations made in veronal-saline buffer. Readings were by the pattern method of Salk after standing at room temperature for 90 minutes, or until patterns were evident in control tubes (more rapid at 37°C, slower at 4°C). Complete or almost complete (+++ or ++++) agglutination was taken as the endpoint. One HA unit is defined as 0.4 ml of the highest dilution which gives complete or almost complete hemagglutination. *Hemagglutination-Inhibition.* Hemagglutination - inhibition (HI) tests were performed by mixing 0.2 ml aliquots of serum or two-fold serum dilutions with 0.2 ml of viral antigen containing 4 HA units and incubating the mixture at room temperature for 2 hours. After serum-virus incubation, 0.1 ml of cell suspension was added to each tube and the occurrence of HA was observed as described above. Endpoints were taken as the serum dilution completely or almost completely preventing agglutination (0 or +). Some animal sera, notably rabbit, possess agglutinins for human Group O

TABLE I. Hemagglutination of Enteric Viruses in Veronal Buffer, pH 7.3, with Human Group O Erythrocytes.

Virus*		HA titer (1:x) at indicated temp. of incubation 8°C	25°C	37°C
ECHO	3	128	64	<2
	4	2	2	2
	6	64	32	<2
	7	2048	2048	2048
	10	32	32	32
	11	256	256	<16
	12	1024	1024	2048
Coxsackie	B_1	+undil.	+undil.	0
	B_2	++undil.	+undil.	0
	B_3	32	32	<2†
	B_4	1	1	1
	B_5	++undil.	++undil.	++undil.

* Also tested at all 3 temperatures but not observed to possess HA activity were preparations of polioviruses 1, 2 and 3, ECHO viruses 1, 2, 5, 8, 9, 13 and 14, and Coxsackie A_9.

† After only 60 min. at 37°C, the Coxsackie B_3 HA titer was usually identical with that at 8°C and 25°C but between 60 and 90 min. the HA pattern usually reversed.

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TABLE II. Hemagglutination Inhibition Titers of Rabbit Hyperimmune Sera.

Virus ↓	Serum→	Reciprocal of H-I titer ECHO 3	6	7	10	11	12	Cox. B_3	ECHO 4
ECHO	3	*4096*	<64	<128	<64	<64	<32	<32	<32
	6	<64	*2048*	<64	"	"	<64	64	<64
	7	"	<64	*1024*	<16	<32	<16	<16	<32
	10	"	"	<32	*4096*	"	"	"	"
	11	"	"	"	<16	*256*	32	"	"
	12	"	"	"	"	<32	*256*	"	"
Cox.	B_3	"	"	<16	<64	<64	<16	*4096*	"

erythrocytes to a titer as high as 1:128. In such cases prior absorption of the serum with 5-10% red cells serves to remove the activity.

Viruses. The stock viruses used here were all prepared in large bottles containing primary outgrowths of trypsinized monkey kidney cells prepared by the usual technics(5). The growth medium consisted of 0.5% lactalbumin hydrolysate and 2% calf serum in Hanks' salt solution, and the confluent sheets of cells were maintained on 0.5% lactalbumin hydrolysate in Hanks' solution.

Results. Virus stocks prepared in monkey kidney cultures were tested for agglutinin activity against Group O erythrocytes at 3 different temperatures as seen in Table I. ECHO viruses 3, 6, 7, 10, 11, and 12 and Coxsackie B_3 exhibited HA activity in dilutions varying from 1:32 to 1:2048. Although significant differences in HA titer were not observed as between reactions carried out at 8°C and at 25°C, the titers obtained at 37°C permitted the clear division of these 7 agents into 2 groups: one (ECHO viruses 7, 10 and 12) with titer unaffected and the other (ECHO viruses 3, 6, and 11 and Coxsackie B_3) with titer greatly depressed. Polioviruses 1, 2 and 3, ECHO viruses 1, 2, 5, 8, 9, 13 and 14 and Coxsackie A_9 were tested at all 3 temperatures but were not observed to possess HA activity. Coxsackie B_1, B_2, B_4, and B_5 and ECHO 4 have exhibited slight HA activity, but since it has not been possible to obtain more potent preparations than those illustrated, further work with these agents has been deferred.

Numerous preparations of the prototype strains of ECHO viruses 3, 6, 7, 10, 11, and 12 and Coxsackie B_3 have been observed to have hemagglutinating activity for human Group O erythrocytes. During the course of these studies, cultures from at least 20 batches of monkey kidney tissue were used, each produced by pooling the kidneys from 3 monkeys. Throughout this time, control culture fluids have never exhibited HA activity. All of 6 Group O donors, including one known Rh negative individual, yielded red cells of comparable activity.

In Tables II and III are summarized the results of HI tests using hyperimmune rabbit sera prepared in this laboratory by Mr. Louis Potash, and monkey sera obtained through the courtesy of Dr. Herbert Wenner. It is to be noted that homologous titers invariably exceeded the heterologous to a significant de-

TABLE III. Hemagglutination Inhibition Titers of Monkey Hyperimmune Sera.

Virus ↓	Serum→	Reciprocal of H-I titer ECHO 3	6	7	10	11	12	4
ECHO	3	>*16,384*	64	128	128	128	64	64
	6	64	*16,384*	"	64	"	128	256
	7	256	128	*4096*	128	"	64	"
	10	128	64	128	>*16,384*	"	32	64
	11	32	128	"	128	*4096*	"	"
	12	64	32	64	64	32	*4096*	"
Cox.	B_3	128	512*	128	128	256	256	512†

* Pre-immunization H-I titer 1:1024. † Pre-immunization H-I titer 1:128.

TABLE IV. Hemagglutination Inhibition Titers of ECHO Antisera from Different Sources.

	Reciprocal of H-I titer				
Serum → / Virus ↓	Rabbit ECHO 7*	Rabbit ECHO 10*	Rabbit ECHO 11*	Rabbit ECHO 6†	Monkey ECHO 7‡
ECHO 7	>1440		40		>1440
10		>1440			40
11	<20	<20	160		
6				512	

* Courtesy of Dr. Albert Sabin.
† " " Dr. David Karzon.
‡ " " Dr. Joseph Melnick.

gree.

HI tests using an anti-E7 monkey serum obtained from Dr. Joseph Melnick, anti-E7, E10 and E11 rabbit sera from Dr. Albert Sabin and anti-E6 rabbit serum from Dr. David Karzon yielded similar results (Table IV). Anti-Coxsackie sera (A_9, B_1, B_3, and B_4), prepared by inoculation of mouse passage material into hamsters, were obtained through the courtesy of Dr. Gilbert Dalldorf. When these were tested with B_3 viral HA, the homologous HI titer (1:4096) proved identical with that of the rabbit antiserum prepared by immunization with virus propagated in monkey kidney cultures and far in excess of the maximum heterologous titer (1:128 with B_4 antiserum) observed.

Fifty-five newly isolated strains including 1 of E10, 7 of E11, 12 of E7, 8 of E12 and 27 of E6 provided by Dr. Wolf Henigst and Mr. Louis Potash of this laboratory have been tested with the uniform finding of hemagglutinating activity, either immediately or after a few passages in monkey kidney cultures. The HA activity of selected examples of the above strains of ECHO 6, 7, 10, 11 and 12 has been shown to be specifically inhibited by immune rabbit serum.

Preliminary data indicate that the hemagglutinins reported here are sedimentable by ultracentrifugation and are removed from the supernatant during the process of agglutination in parallel with infectious virus. They are active over a wide range of pH, yielding a fairly constant HA titer in the range of 7.0 to 8.0. Preliminary studies of the hemagglutinin-erythrocyte reaction indicate that hemagglutinin can be eluted from the red cell and that the latter may be so altered as to be inagglutinable by homologous virus. Such virus-treated erythrocytes appear to exhibit a receptor gradient roughly in the order of E6, Coxsackie B_3, E3, E11, E7, E12, and E10. Despite analogies between these findings and some of the characteristics of the myxovirus group, treatment of erythrocytes with Receptor Destroying Enzyme (R D E) or influenza B virus (Lee) appears to result in no decrease of their reactivity with the enteric hemagglutinins and, conversely, enteric virus-treated erythrocytes appear to remain fully reactive with influenza A′ virus (Malaya).

HI titrations of paired human sera collected in relation to infection with Coxsackie B_3 and ECHO viruses 6, 7, 10, 11 and 12 have been performed with the frequent demonstration of a rise in HI titer in the later specimen. The phenomenon of non-specific inhibition, however, has been encountered in both human and animal sera. Until a reliable method is found for removing these inhibitors, the results of HI tests must be interpreted with care.

Discussion. The knowledge that so-called simian viruses are frequently found as contaminants in cultures of monkey kidney cells and that some of these agents possess the ability to agglutinate human Group O erythrocytes(3) should lead to a healthy skepticism when the phenomenon is noted in preparations of enteric viruses grown in this tissue culture system. Indeed, since virus stocks may be contaminated by simian agents, antisera prepared by hyperimmunization of animals with such stocks may conceivably contain HI activity for the contaminant.

The following evidence is available to indicate that the hemagglutinins described in this report are specifically related to the enteric agents, rather than to an hypothetical contaminant. 1) Newly isolated strains of ECHO viruses 6, 7, 10, 11 and 12 have been observed to have HA activity which is specifically inhibited by homologous hyperimmune sera. 2) Hyperimmune sera prepared in 4 different laboratories were found to have HI activity. In the case of Coxsackie B_3, antiserum prepared with mouse passage material was fully as active as that prepared by the inoculation

of virus propagated in monkey kidney cultures. 3) Attempts have been unsuccessful to separate a hypothetical simian contaminant by adaptation of these agents to HeLa, KB and propagable amnion cells (to be reported later) and serial passage in these cultures. 4) Humans infected with these enteric agents (except for ECHO 3 and 10) have been studied serologically, and a substantial number have shown significant rises in HI antibody titers in the post-infection specimens.

Separation of the viruses into 2 groups according to the influence of 37°C incubation on the HA titer results in an interesting parallelism with the grouping of the ECHO viruses proposed by Hsiung and Melnick(6) on the basis of plaque morphology and the susceptibility of patas and rhesus kidney cells. The proposed ECHO virus group A contains all 3 of the ECHO viruses (3, 6, and 11) with HA titers depressed at 37°C while group B contains 2 (E7 and E12) of the 3 viruses with HA titers unaffected by the temperature of incubation. The third member of this latter group (E10) was not assigned to either group A or group B.

Summary. 1) Monkey kidney cell culture fluids containing ECHO viruses 3, 6, 7, 10, 11 and 12 and Coxsackie B_3 have been shown to be capable of agglutinating human Group O erythrocytes in relatively high dilutions. Evidence has been presented indicating that the hemagglutinating activity is specifically related to these agents, rather than to possible simian viral contaminants. 2) Preliminary findings suggest that the HA property resides in the viral particle, that it is absorbed by erythrocytes during the process of agglutination and may be eluted from them, exhausting red cell receptors during the process. Despite such similarities to the myxoviruses, additional data suggest that these agents do not belong to the myxovirus group. 3) Monkey kidney cell preparations of polioviruses 1, 2, and 3, ECHO viruses 1, 2, 5, 8, 9, 13 and 14 and Coxsackie A_9 failed to agglutinate human Group O cells, whereas preparations of ECHO 4 and Coxsackie B_1, B_2, B_4 and B_5 manifested HA activity in low dilution.

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Effects of a Quaternary Derivative of Atropine, N-Benzyl Atropinium Chloride. (23610)

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We have shown(1) that N-benzyl and N-phenacyl atropinium salts, as well as certain other compounds containing quaternary nitrogen atoms, are capable of overcoming the Sarin-induced decrease in twitch response of the indirectly stimulated gastrocnemius-soleus-tibialis anticus muscle group of the cat. The present work was undertaken to study some of the functional changes induced by these compounds.

Methods. Cats, rabbits and guinea pigs of unselected sex were used. Young, male mice weighing about 20 g were used in toxicity determinations. LD_{50} values were calculated by the method of Miller and Tainter(2). Cats and rabbits were anesthetized with sodium pentobarbital (30 mg/kg i.p. or i.v., respectively). A metal bellows manometer recorded blood pressure; intratracheal pressure was recorded from a side-arm cannula by a Marey tambour. ECG was recorded with a Sanborn Viso-Cardiette and EEG by a Grass Electro-