ASSOCIATION OF A NEW TYPE OF CYTOPATHOGENIC MYXOVIRUS WITH INFANTILE CROUP*

By ROBERT M. CHANOCK, M.D.

(From the Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati)

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At the present time most infectious croup in infancy, seems to be unrelated to *Corynebacterium diphtheriae* or *Hemophilus influenzae* B; moreover recent studies have failed to implicate other pathogenic bacteria (1). The term "viral croup" has been applied to this syndrome without the necessary justification of an etiologically associated viral agent. The purpose of the present study was to determine whether cytopathogenic agents could be isolated from infants with croup and, if so, to assess their importance in the causation of the observed disease. During the course of this work 2 viruses, which produced an unusual cytopathogenic effect, were isolated in tissue culture of monkey kidney epithelium. Characterization of these viruses, which appear to be new members of the myxovirus group, forms the basis of the present report.

Materials and Methods

Tissue Cultures.—Cynomolgus monkey kidney epithelial cultures were used in all experiments except for the instances noted in the text. Monkey kidney cultures consisted of an outgrowth of trypsinized cells in roller tubes containing 0.5 ml. of 0.5 per cent lactalbumin hydrolysate in Hanks' solution and 2 per cent heated calf serum. Six to 7 days after the tubes were prepared 1.5 ml. of 0.5 per cent lactalbumin hydrolysate in Earle's solution was added. The cultures, which contained a final serum concentration of 0.5 per cent, were then ready for use. Human amnion cultures were prepared in a similar manner except that 20 per cent heated calf serum was incorporated in the medium.

Specimens for Virus Isolation.—Throat swabs from infants with croup and from infants with non-respiratory illness were immersed in 5 ml. of Hanks' solution containing 2,000 units of penicillin, 2,000 μg. of streptomycin, and 150 units of mycostatin per ml. Specimens were tested immediately or after storage at −20°C. for several days. 0.2 ml. of throat swab fluid was inoculated into each of 3 to 5 monkey kidney epithelial cultures and the nutrient medium (0.5 per cent lactalbumin hydrolysate in Earle's solution) changed every 4 to 5 days when the pH decreased to 6.6 to 6.8. Inoculated cultures were observed daily for 18 to 23 days, the length of time depending on the viability of the cell sheet.

Infectivity Titrations and Neutralization Tests in Tissue Culture.—Titration of infectivity was performed by inoculating 0.2 ml. of decimal dilutions of the test suspension into each of

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3 roller tubes of monkey kidney. The cultures were observed daily from the 3rd to 8th post-inoculation day. In the neutralization test, fourfold dilutions of unheated serum and an equal volume of virus estimated at 100 TCD_{50} (50 per cent tissue culture doses) were incubated for 1 hour at room temperature and then inoculated in 0.2 ml. amounts into each of 3 monkey kidney cultures. Simultaneous titration of the virus employed was included in every neutralization test. The final readings were made on the 6th day after inoculation and the serum $titer_{50}$ (50 per cent serum dilution end point) calculated at that time by the method of Reed and Muench.

Hemagglutination.—Unless otherwise stated hemagglutination tests were performed with 0.25 per cent newborn chick erythrocyte suspensions. Equal volumes (0.5 ml.) of serial twofold dilutions of the material to be tested for hemagglutinin activity and erythrocyte suspension were shaken and allowed to sediment for 1½ hours at 4°C. Red cells from 24 to 72 hour old chicks were obtained and processed as described previously (2). The diluent for the suspension to be tested for hemagglutinins was 0.9 per cent NaCl solution, either unbuffered or buffered at pH 8.0 with 0.02 M sodium borate-boric acid (3).

Hemagglutination-inhibition (H-I) tests were performed with 0.25 ml. serial twofold dilutions of inactivated (56°C. for 30 minutes) serum and an equal volume of hemagglutinin diluted to contain 4 units. As in the hemagglutinin titrations, newborn chick erythrocytes were added as a 0.25 per cent suspension in a volume of 0.5 ml.

Complement-Fixation.—The C-F technique of Schmidt and Lennette (4) was employed with the modification that 2 to 4 units of antigen were used rather than the single unit described for this method. Briefly, twofold dilutions of serum (0.2 ml.), 2 to 4 units of antigen (0.2 ml.), and 2 exact units of complement (0.2 ml.) were incubated overnight at 4°C. after which 2 per cent sheep red cells sensitized with 2 units of hemolysin (0.5 ml.) was added and the system incubated for 30 minutes at 37°C. Complement titrations were performed in the presence of the antigen. The highest dilution of serum which exhibited 75 per cent or greater fixation of complement was considered the end point.

Serum Collection and Storage.—All blood specimens were placed in silicone-treated centrifuge tubes and the clot allowed to retract for not longer than 16 to 18 hours at 4°C. The separated serum was stored in rubber stoppered ampules at −70°C. in a dry ice chest.

Immune Sera.—Potent immune serum for one of the agents isolated in this study was prepared by intravenous inoculation of *cynomolgus* monkeys with 1 ml. of infected tissue culture fluid (10^6TCD_{50}); intravenous inoculations were repeated after 7, 14, 21, and 32 days. The monkeys were bled out 10 days after the last inoculation.

Immune Serum for Other Viruses.—Specific antiserum for various viruses was kindly supplied by the following investigators: rooster sera for influenza A, A′, B, and C viruses by Dr. M. R. Hilleman, human and guinea pig sera for mumps virus by Drs. Gertrude and Werner Henle, monkey serum for Sendai virus and human sera for influenza by Dr. K. Jensen, chicken serum for Newcastle (NDV) virus by Dr. F. Bang, rabbit serum for the non-APC virus isolated in California from adults with respiratory disease (5) by Dr. T. Berge, rabbit sera for the simian (SV) viruses by Dr. R. Hull (6), rabbit serum for an agent associated with rhinitis in chimpanzees by Dr. A. B. Sabin (7), chimpanzee serum for another distinct agent associated with rhinitis in chimpanzees by Drs. J. A. Morris and J. E. Smadel (8), ferret serum for distemper by Dr. H. Koprowski, and rabbit serum for herpes simplex by Dr. I. Ruchman.

Receptor-Destroying Enzyme (RDE).—RDE derived from *Vibrio cholerae* culture filtrate was kindly supplied by Dr. Oscar Liu. RDE activity was titrated by the method of Burnet and Stone (9). The diluent used in these titrations was the calcium acetate saline buffer pH 6.2 described by Ada and Stone (10). The preparation of RDE used in all the experiments titered 1:256 to 1:512 with one exception when tested with 8 units of FW-1-50 influenza A′ virus.

RDE Treatment of Serum.—Equal volumes of undiluted serum and RDE were incubated for 15 hours at 37°C., after which the mixture was inactivated at 56°C. for 30 minutes. Simultaneous titrations of RDE potency were performed with each experiment in which sera were treated.

Gradocol Membrane Filtration.—Undiluted fluid from tissue cultures infected with one of the agents recovered in this study, with a potency of $10^{5.4}$ to $10^{6.2}$ TCD_{50} per ml., was first clarified by serial filtration through 970, 650, and 480 mμ gradocol membranes. The 480 mμ filtrate was then filtered through a 320, 286, 247, 197, 180, 93, or 58 mμ membrane. One ml. of filtrate was inoculated into each of 10 monkey kidney culture tubes.

RESULTS

Isolation of Viruses from Infants with Croup

Cytopathogenic agents were isolated from the throat swabs of 2 of 12 infants with the diagnosis of infectious croup (acute laryngotracheobronchitis). These patients ranged in age from 3 to 30 months. As shown in Table I virus isolation

TABLE I

Incidence of CA Virus Isolation and Serologic Evidence of Infection in Infants (a) with Croup and (b) with Non-Respiratory Illness

Date	Croup		Non-respiratory	
	Virus isolation	Antibody rise*	Virus isolation	Antibody rise*
Oct., 1955	0/3	2/3	—	—
Nov., 1955	2/4	3/4	—	—
Dec., 1955	0/4	0/3	0/8	1/8
Jan., 1956	0/1	0/1	0/8	0/8
Total.....	2/12	5/11	0/16	1/16

* As determined by tests with paired sera employing the monkey kidney tissue culture neutralization technique and the H-I procedure with RDE-treated serum.

attempts were successful only during the 2nd month of the study (November). After the successful isolations in the initial 2 month pilot study a control group of infants with non-respiratory illness was instituted. Unfortunately the control infants were studied at a time when infants with croup failed to yield cytopathogenic agents and failed to exhibit serologic evidence of infection with the agents isolated earlier; *i.e.*, in November.

Based on virus isolation and antibody rise to the isolated viruses, 5 of the 7 infants with croup in October and November were infected with the recovered agents, while the remaining 5 infants ill with croup in December and January were not. It is of interest that 1 infant with non-respiratory illness developed a definite antibody rise to the agents isolated from the croup patients.

Although pneumonitis was clinically recognizable in only 1 of the infants with croup—a virus was isolated from this infant—x-ray examination revealed definite and, in some in-

stances, extensive pneumonic infiltration in 4 and possibly 5 of the patients. The incidence of pneumonitis was the same for infants with or without evidence of infection with the recovered agents, 2 and possibly 3 of 5 in the former and 2 of 5 in the latter group. Pneumococcus (not typed) was isolated from the nose and throat culture of 1 infant in the group infected with the recovered agents while beta hemolytic streptococcus was recovered from an infant in the other group. Nose and throat cultures of the remaining croup patients did not contain pathogenic bacteria. *H. influenzae* B was not encountered.

Properties of the Isolated Viruses

Behavior in Tissue Culture.—On primary isolation in monkey kidney epithelial cultures cytopathogenic changes were not observed until the 10th and 15th day after inoculation of the 2 positive throat swab fluids. The incubation period shortened to 3 to 5 days during the second culture passage, while virus from subsequent passages regularly produced observable changes by the 3rd to 4th day with limiting infective doses requiring 5 to 6 days and rarely 7 to 8 days. Infected tissue culture fluid regularly contained 10^5 to 10^6 TCD_{50} per ml.

The first change seen in infected cultures was the formation of a syncytial area with loss of cell boundaries, rapidly followed by a breaking away from the surrounding cell sheet. Numerous small vacuoles made their appearance within the cytoplasm of the cells, and together with the increased granularity which accompanied the loss of discernible cell structure produced a picture best described as "sponge-like" (Figs. 1 to 4). Within 24 to 48 hours of the first change 80 to 90 per cent of the epithelial sheet was affected and off the glass, while the remaining normal appearing epithelium persisted in a "Swiss cheese" like pattern. A similar sequence of events followed inoculation of human amnion cultures, which in a simultaneous test proved to be 10 times more sensitive than monkey kidney cultures in the detection of minimal quantities of virus.

The sponge-like appearance of the cytopathogenic change just described has not been observed with other viruses studied in this laboratory (poliomyelitis, ECHO viruses both classified and unclassified (7, 11), chimpanzee rhinitis (7), herpes simplex, pseudorabies, B virus, mumps, and NDV (12)) and appears to be a unique feature of the agents isolated in this study. These agents will be referred to as CA viruses, signifying "croup associated" although the ultimate role of these viruses in infantile croup remains to be elucidated by further studies.

Physical Properties.—The "Greer" strain of CA virus passed through a 197 mμ but not a 180 mμ gradocol membrane. Employing the formula of Elford (13) the size by ultrafiltration was estimated to be 90 to 135 mμ. The CA viruses were stable at −70°C. for at least 5 months. Overnight storage of the "Greer" strain of CA virus at 4°C. did not affect its potency while exposure to 20 per cent ether for 15 hours at 4°C. resulted in a complete loss of infectivity.

Hemagglutination.—Fluid from infected monkey kidney cultures agglutinated chick erythrocytes and in lower titer human "O" red cells as shown in Table II.

Highest titers were obtained when the hemagglutinin dilutions were performed in 0.02 M borate saline buffered at pH 8.0 as shown in Table III and the chick erythrocytes allowed to

sediment at 4°C. (Table II). Chick red cells were capable of adsorbing 88 per cent of the hemagglutinin from the culture fluid at 4°C., while complete elution took place at 37°C. as

TABLE II

Effect of Erythrocyte and Temperature of Sedimentation upon Potency of CA Hemagglutinin

Temperature of sedimentation	Reciprocal of titer* with indicated 0.25 per cent erythrocyte suspension	
	Newborn chick	Human "O"
°C.		
4	32	4
23	16	4
37	1	1

* Diluent was 0.02 M sodium borate-boric acid in 0.9 per cent NaCl pH 8.0.

TABLE III

Effect of pH upon Hemagglutination by CA Virus

Diluent	pH	Titer
0.9 per cent NaCl	—	8
0.02 M phosphate in 0.9 per cent NaCl	6.0	4
" " " " " " " "	6.4	4
" " " " " " " "	6.8	2
" " " " " " " "	7.2	8
" " " " " " " "	7.6	8
0.02 M sodium borate-boric acid in 0.9 per cent NaCl	8.0	16

Buffers prepared and checked electrometrically as described previously (3). 0.25 per cent chick erythrocyte suspension and sedimentation at 4°C. were employed.

TABLE IV

Adsorption on and Elution from Chick Erythrocytes of CA Hemagglutinin

Material tested	Titer of hemagglutinin*
Original tissue culture fluid	64
Supernate after adsorption with an equal volume of 5 per cent chick red cells at 4°C. for 2 hrs.	8
Supernate of resuspended red cells, 2 hrs. at 37°C.	64

* Expressed in terms of original culture fluid.

shown in Table IV. Within 10 minutes after agglutinated red cells were placed at 37°C. there was a complete reversal of agglutination. However, when such cells were resuspended and allowed to sediment at 4°C. the original positive pattern was restored. Five successive cycles of agglutination at 4°C. and disagglutination at 37°C. were carried out with mixtures containing 1 to 32 units of hemagglutinin. It was possible to restore the original agglutination patterns when hemagglutinin (2 to 64 units)-red cell mixtures which had been held at 37°C.

for 20 hours were resuspended and allowed to sediment at 4°C. Hemagglutinin in the absence of red cells was stable over a 20 hour period at 37°C.

The picture of the hemagglutinin-chick erythrocyte reaction which emerges from these observations is one of a reversible temperature-dependent phenomenon with adsorption and agglutination occurring at 4°C. and dissociation and reversal of agglutination resulting from exposure to 37°C.

TABLE V

Evidence that CA Hemagglutinin Modifies Its Receptors on the Chick Erythrocyte

Exp.	Cells treated 24 hrs., 37°C.*	Pattern of hemagglutination produced by indicated virus									
		CA								4 units of PR-8, FM-1, FW-1-50, FLW-1-52, Lee, IBI, or NDV	Diluent only
		Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
1	Normal tissue culture fluid			++	++	++	++	±		++	0
	CA HA-32 units			±	±	±	±	±		++	0
2	Normal tissue culture fluid		++	++	++	++	+	0	0	++	0
	CA HA-32 units	++	++	++	+	±	0	0	0	++	0
3‡	Normal tissue culture fluid		++	++	++	++	++	±	0	++	0
	CA HA-32 units	++	++	++	±	0	0	0	0	++	0

* Equal volumes of 0.5 per cent chick erythrocyte suspension and either normal or infected tissue culture fluid incubated for 24 hours at 37°C. Chick red cells then washed 2 to 3 times with 5 ml. of 0.9 per cent NaCl and prepared as 0.25 per cent suspension in 0.9 per cent NaCl.

‡ First washing of erythrocytes performed with 5 ml. of a 1:80 dilution of CA monkey hyperimmune serum.

In addition to the reactions just described, CA hemagglutinin was capable of partially removing its receptor sites on the chick erythroycte.

This weak enzymatic effect was demonstrable only when maximal concentrations of hemagglutinin (32 units) were allowed to incubate with chick red cells for 24 hours at 37°C. and the treated cells tested with lesser concentrations of hemagglutinin in the 1 to 8 unit range (Table V). When treated cells were tested with larger amounts of hemagglutinin complete agglutination patterns were produced, explaining why receptor modification was not observed in the resuspension experiments described above.

Chick erythrocytes whose CA receptors were partially removed by the action of CA virus remained fully agglutinable by influenza A and B and Newcastle virus hemagglutinins.

"Receptor destroying enzyme" (RDE) of *Vibrio cholerae* filtrate removed the receptors for the CA hemagglutinin from the chick erythrocyte.

The quantity of RDE required to render chick red cells inagglutinable by CA hemagglutinin was the same or slightly less than that required for an influenza A′ strain (FW-1-50) which was tested simultaneously. In 2 comparative tests the titer of RDE was 1:256 and 1:64 against 8 units of FW-1-50 influenza A′ virus and 1:256 or greater (no end point) and 1:128 against 8 units of CA virus.

RDE and $NaIO_4$ removed most or all of the non-specific inhibitor for CA hemagglutinin present in certain sera, thus permitting use of the hemagglutination-inhibition (H-I) technique for assay of specific antibody (Table VI).

Host Range.—Multiplication of the "Greer" strain of CA virus occurred in the amniotic cavity of the 9 to 10 day fertile hen's egg when a 5 day incubation period was employed (Table VII). Apparently growth occurs at a slow rate in the amniotic cavity since propagation could not be demonstrated when passages were performed at 3 day intervals. The quantity of virus produced in the

TABLE VI

Effect of RDE and $NaIO_4$ upon Inhibitor for CA Hemagglutinin Present in Certain Sera

Serum	H-I titer after indicated treatment			
	None	RDE*	None	$NaIO_4$‡
Normal rabbit A	80	<5	160	40
Monkey 92-65				
Pre-immunization	80	5	160	20
Post immunization with CA virus	320	320	640	640

* Incubation of equal volumes of undiluted serum and undiluted RDE (titer of 1:256) for 15 hours 37°C. Serum-RDE mixture then inactivated 56°C. for 30 minutes.

‡ Incubation of 0.25 ml. undiluted inactivated serum and 0.15 ml. 0.1 M $NaIO_4$ for 4 hours 37°C. 0.15 ml. 40 per cent glucose then added to stop the action of the periodate.

amniotic cavity was not sufficient to agglutinate chick erythrocytes. Employing a 3 day incubation period multiplication did not occur in the allantoic cavity. Pock formation was not observed 3 days after inoculation of the chorioallantoic membrane with decimal dilutions of virus ranging from 10^1 to 10^5 TCD_{50}.

The "Greer" strain of CA virus was not pathogenic for suckling (1 day old) or weanling mice by intracerebral or other parenteral routes of inoculation. Intranasal instillation of $10^{4.7}$ TCD_{50} of virus failed to produce lung lesions in weanling mice and a study of lung tissue removed after various intervals indicated that virus did not multiply.

Antibody for the CA Viruses in Various Human Sera

H-I antibody levels in convalescent sera from croup patients were low when the conventional H-I technique of adding hemagglutinin and erythrocytes in rapid succession to dilutions of serum was employed. The sensitivity of the

H-I procedure was increased considerably when it was discovered that incubation of immune serum with hemagglutinin for 2 hours at room temperature, before the addition of red cells, resulted in a fourfold increase in serum titer as shown in Table VIII. Based on these findings this technique was adopted as a standard procedure.

TABLE VII

Propagation of CA Virus in the 9 to 10 Day Old Embryonated Egg

Passage	Quantity of virus or hemagglutinin recovered					
	Allantoic inoculation*		Amniotic inoculation*			
	3 day incubation		3 day incubation		5 day incubation	
	Virus TCD_{50} per ml. allantoic fluid‡	Hemagglutinin	Virus TCD_{50} per ml. amniotic fluid	Hemagglutinin	Virus TCD_{50} per ml. amniotic fluid	Hemagglutinin
1	$10^{3.2}$	0§	$10^{1.2}$	0	$10^{2.2}$	0
2	$<10^{0.7}$	0	$<10^{0.7}$	0	$10^{3.5}$	0
3			$<10^{0.7}$	0	$10^{4.5}$	0

* Original inoculum = 10^5 TCD_{50}.

‡ As determined in monkey kidney tissue culture.

§ 0.5 ml. of undiluted fluid failed to hemagglutinate an equal volume of 0.25 per cent chick erythrocytes.

TABLE VIII

Effect of Incubation of Hemagglutinin-Serum Mixtures at 22°C. on the Titer of Hemagglutination-Inhibition Antibody

Serum	Reciprocal of H-I titer following indicated manipulation	
	Immediate addition of chick RBC to HA-serum mixtures	Incubation of HA-serum mixtures 2 hrs. 22°C. before addition of chick RBC
Croup patient La.		
5 days after onset	<5	<5
58 days after onset	40	160

Antibody Response of Infants with Croup.—The 2 infants from whom the CA viruses were isolated responded with significant rises in H-I, C-F, and neutralizing antibody during convalescence (Table IX). Three additional infants with croup, from whom no virus was isolated, exhibited a rise in one or more of the 3 varieties of antibody. The limited neutralizing antibody rise developed by patient Pur. presumably reflects the early period in convalescence when the second serum specimen was collected. Interpretation of the antibody response in this instance is made with caution in view of the question of specificity of neutralizing titers of 1:16 or less as will be described later.

TABLE IX

Antibody Response of Infants with Croup to CA Viruses Isolated from Patients Greer and Lorentz

Category	Patient	Age	Days after onset	Reciprocal of antibody titer with indicated strain			
				Greer			Lorentz
				H-I*	C-F	Neut‡	Neut§
		mos.					
Virus isolated. Neut, H-I, and C-F rise.	Greer	11	3	<5	<4	<2	16
			23	80	64	64	48
			107	80			
	Lorentz	4	5	<5	<4	8	8
			32	80	64	64	32
No virus isolated. Neut, and/or H-I and/or C-F rise.	Cro.	3	4	<5	<4	24	
			28	20	16	42	
			60	20			
	Pur.	16	4	<5		<2	<2
			12	<5		12	10
	La.	22	5	<5	<4	8; 4	<2
			10			10	
			58	160	64	256	128
			101	80			
No virus isolated. No antibody rise.	Cl.	30	1	<5		16	
			53	<5		10	
	Sh.	19	2	<5		16	16
			19	<5		10	16
	Ev.	9	4	40		64	
			30	40		64	
	Ot.	24	4	<5		4	
			29	<5		<2	
	Mo.	18	5	<5	<4	8	12
			25	<5	<4	10	10
	Th.	29	8	<5	<4	4	
			26	<5	<4	4	

* Highest original dilution of RDE-treated serum which produced complete or almost complete inhibition of 4 units of hemagglutinin.

‡ Serum $titer_{50}$ *vs.* 32 to 320 TCD_{50} of virus in monkey kidney tissue culture.

§ Serum $titer_{50}$ *vs.* 64 TCD_{50} of virus in monkey kidney tissue culture.

Fourfold or greater rises in H-I, C-F, or neutralizing antibody were not encountered during convalescence of the remaining 6 infants with croup. One of these infants (patient Ev.) possessed high unchanging levels of both H-I and neutralizing antibody.

Antibody Pattern of Control Group.—A study of serum specimens obtained from infants with non-respiratory illness (gastroenteritis, surgical conditions, etc.) at the time of hospitalization and 3 to 5 weeks later revealed that only 1 of the 16 infants developed a significant (fourfold) rise in H-I or neutralizing antibody. The antibody values for this infant (diagnosis of chronic otitis media) at the time of hospitalization, 1 month later, and 2 months later were: serum neutralization $titer_{50}$ of <1:2, 1:16, and 1:42, and H-I antibody of <1:5, 1:10, and 1:40. At no time during this period did the infant manifest any respiratory symptoms.

TABLE X

Incidence of H-I Antibody for CA Virus in Certain Human and Animal Sera

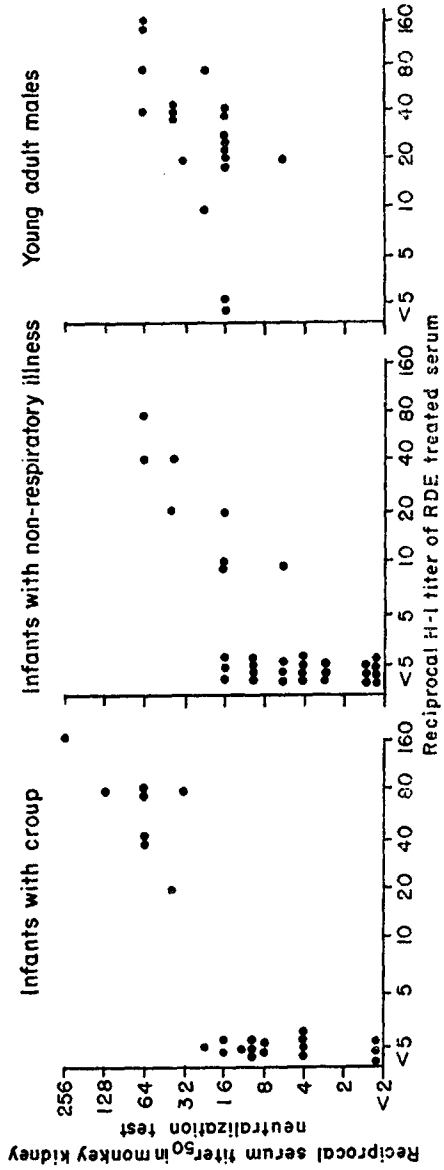
Species	Category	No. tested	Positive*	Positive titer	
				Range	Mean
			per cent		
Human	2½–34 mos.	16	25	10–80	38
	21–30 yrs.	20	90	10–160	48
Chimpanzee	Normal	7	0	—	—
Cynomolgus monkey	Normal	10	0	—	—
	After immunization with CA virus	4	100	160–640	360

All sera except those of chimpanzees were treated with RDE. Five chimpanzee sera were tested untreated while the remaining 2 were tested after $NaIO_4$ treatment.

* H-I titer of 1:10 or greater.

Incidence and Significance of H-I Antibody.—Confidence in the specificity of H-I antibody was established by several lines of evidence: the development of this antibody by infants from whom CA viruses were isolated (Table IX), its absence from the serum of normal monkeys and chimpanzees and its high level in the serum of immunized monkeys (Table X), and lastly the excellent correlation of its presence in various human sera and a serum neutralizing $titer_{50}$ of 1:16 or greater. As seen in Text-fig. 1 with one exception, a serum-neutralizing $titer_{50}$ of greater than 1:16 was accompanied by H-I antibody and, conversely, with 2 exceptions the presence of H-I antibody was associated with a serum neutralizing $titer_{50}$ of 1:16 or greater.

The incidence of H-I antibody in infants with non-respiratory illness (25 per


Infants with croup
Infants with non-respiratory illness
Young adult males
Reciprocal serum titer50 in monkey kidney neutralization test
256
128
64
32
16
8
4
2
<2
<5
5
10
20
40
80
160
Reciprocal H-I titer of RDE treated serum

TEXT-FIG. 1

cent) and in young adult males (90 per cent) strongly suggests that appreciable infection with CA virus occurs early in life and during childhood.

Significance of Serum Neutralizing Activity.—On the basis of the available evidence a serum neutralization $titer_{50}$ of greater than 1:16 would appear to represent specific neutralizing activity. Thus, such levels were regularly found (*a*) in the convalescent sera of infants with croup from whom CA viruses were isolated or who developed H-I and C-F antibody rises (Table IX) and (*b*) in the sera of monkeys immunized with CA virus (Table XI). In addition, the correlation of H-I antibody and a serum neutralization $titer_{50}$ of greater than 1:16 further supports this assumption (Text-fig. 1).

TABLE XI

Neutralizing Titer for CA Virus of Certain Human and Animal Sera

Category	No. with indicated serum $titer_{50}$*										Total
	<2	4	6	10	16	24	32	42	64	128	
Human											
2½–34 mos.—no respiratory illness‡	2	2	2	3	3			2	1		15
21–30 yrs.—no respiratory illness			1		9	2	1	3	4		20
Chimpanzee	7										7
Cynomolgus monkey											
Normal	1			2	7						10
After CA virus immunization							2			2	4

* As determined in monkey kidney tissue culture.

‡ Recorded values represent highest serum $titer_{50}$ obtained in tests performed on serum drawn at time of hospitalization and again 3 to 5 weeks later.

The specificity of neutralizing levels of 1:3 to 1:16 is in doubt at the present time since they are found in the sera of 90 per cent of normal monkeys (Table XI) and because neutralizing titers of less than 1:16 in human serum are not associated with the presence of H-I antibody. It is noteworthy that none of the 7 chimpanzee sera tested exhibited any neutralizing activity.

As shown in Table XII inactivation at 56°C. for 30 minutes could not be used to determine the specificity of neutralizing activity in human serum. Such treatment, although completely removing low level neutralizing activity, produced a ten- to sixteenfold reduction in potency of serum containing specific antibody. In contrast, the titer of monkey hyperimmune serum was unaffected by inactivation while the low level neutralizing activity of normal monkey serum was removed.

The results of inactivation of human immune serum suggested that a "heat-labile accessory factor" may be operative in the action of naturally formed

human neutralizing antibody. This hypothesis was tested by adding fresh normal chimpanzee serum free of neutralizing activity to heated human immune serum—the final concentration of the chimpanzee serum after the addition of virus was 1:2 to 1:4. A partial restitution of lost neutralizing activity was observed with 1 of the 4 sera tested; croup infant number 2 (Table XII) possessed a serum neutralizing $titer_{50}$ of 1:32 unheated, 1:2 or less heated, and 1:8 after addition of chimpanzee serum to heated serum. In the other 3 in-

TABLE XII

Effect of Heating at 56°C. for 30 Minutes upon Neutralizing Activity of Certain Human and Monkey Sera

Species	Category	H-I antibody	Reciprocal serum neutralization $titer_{50}$	
			Unheated	Heated
Human	Non-respiratory illness			
	Infant 1	<5	16	1 or <
	2	<5	16	1 or <
	3	<5	12	1 or <
	4	20	21	2 or <
	5	80	32	2 or <
	Croup			
	Infant 1	80	128	8
	2	80	32	2 or <
Cynomolgus monkey	Pre-immunization			
	Monkey 1	<5	4	1 or <
	2	<5	10	1 or <
	6 wks. post immunization with CA virus			
	Monkey 1	640	64	96
	2	320	64	42

stances (croup infant 1 and infants 4 and 5 without respiratory illness) the partial restitution of lost neutralizing activity was a temporary phenomenon, observable only on the 4th and 5th day after inoculation of the culture tubes but not on the 6th day when the final readings were recorded. These findings are only suggestive and do not establish the existence of a "heat-labile accessory factor" for human neutralizing antibody.

Relationship of the 2 CA Viruses to Each Other

Of the 2 CA viruses isolated, the "Greer" strain was studied more extensively. However, when certain properties of the "Lorentz" strain were in-

TABLE XIII

Relationship of CA Virus to Certain Members of the Myxovirus Group and to the RI-APC Group of Viruses

Serum	Reciprocal of antibody titer with indicated virus																
	CA		Influenza												Senadi	NDV	RI-APC
			A		A′				B			C					
			PR-8		FM-1	FW-1-50	FLW-1-52	Spirup	Lee		IBI	1233					
	H-I	C-F	H-I	C-F Soluble	H-I	H-I	H-I	C-F Soluble	H-I	C-F Soluble	H-I	H-I	C-F Soluble	C-F Viral	H-I	H-I	C-F
CA																	
Human																	
Patient Gr. 3 day	<5	<4	<8			<8*			<8		<8	<8					<5
23 "	80	64	<8			<8*			<8		<8	<8					<5
Patient Lor. 5 day	<5	<4	<8			<8*			<8		<8	<8					<5
32 "	80	64	<8			<8*			<8		<8	<8					<5
Patient La. 5 day	<5	<4	<8			<8*			<8		<8	<8					<5
58 "	160	64	<8			<8*			<8		<8	<8					<5
Monkey No. 1																	
Preimmunization	<5	<4		8				8		<8			16	<8			<5
3 wks. postimmunization	320	256		8						<8							
6 " " "	640	256						8		<8			16	<8			<5

Influenza																	
Rooster A PR-8	<10*		1600*														
" A′ FM-1	<10*				1600*												
" " FW-1-50	20					800*											
G. pig " "	<5					1280											
Rooster " WRU-1-50																	
preimmunization	<5					20*											
post "	5					1280*											
" " FLW-1-52	<10*						800*										
" B Lee	<10*								800*								
" " IBI	<10*										800*						
" C 1233	<10*											1600*					
Sendai																	
Monkey normal	<5														<32		
immune	<5														2048		
NDV																	
Chicken normal	5*															<5*	
immune	5*															640*	

* H-I test performed with serum which was not RDE-treated.

C-F tests with the RI-APC group C-F antigens (RI-67, APC types 3 and 7) kindly performed by Dr. Maurice Hilleman. C-F tests with the soluble antigens of influenza virus kindly performed by Drs. Gertrude and Werner Henle and Dr. Edwin H. Lennette.

vestigated they were identical in every instance with those of the "Greer" strain. These properties include; cytopathogenic effect, incubation period, and titer in monkey kidney epithelial culture; reversible type hemagglutination reaction with chick erythrocytes; and antigenic character. Antigenic similarity was established by the identical reaction of Greer and Lorentz hemagglutinins with sera obtained from monkeys before and after immunization with the "Greer" strain. The results of neutralization tests with the 2 strains and paired sera from croup patients shown in Table IX supports this conclusion.

Relationship of CA Viruses to Other Viruses

Relationship to Myxovirus Group.—The CA viruses possess all the properties required for classification in the recently established myxovirus group (14). These properties are: hemagglutination of fowl erythrocytes, viral receptor-destroying enzyme, removal of erythrocyte receptors by RDE of *Vibrio cholerae* filtrate, removal of "normal inhibitor" from serum by RDE, growth in the amniotic cavity of the fertile hen's egg, a size of 80 to 150 mμ, ether sensitivity, and stability at −70°C.

Evidence that the CA viruses are not antigenically related to the influenza viruses is presented in Table XIII.

Convalescent sera from croup patients, which contained high levels of CA H-I antibody, failed to inhibit the hemagglutinins of the influenza A, A′, B, or C strains employed. A monkey which developed a 128-fold homologous C-F antibody rise following immunization with CA virus, failed to exhibit a rise for the group specific soluble C-F antigens of influenza A, B, or C. In addition, potent specific antisera against various influenza prototype strains failed to inhibit CA hemagglutinin. The one exception was the low level inhibition of CA hemagglutinin by a rooster FW-1-50 antiserum; unfortunately the preimmunization serum from this fowl was not available. The significance of this finding was doubtful since a potent FW-1-50 guinea pig antiserum and a rooster antiserum prepared against the antigenically identical WRU-1-50 strain failed to inhibit CA hemagglutinin.

In addition to the data shown in Table XIII, tests with paired sera from 6 adults naturally infected with influenza A′ (both individuals infected in 1956), B (both individuals infected in 1952), or C (infected in 1952 and 1954) supported the conclusion that the CA viruses are distinct from the influenza viruses. These patients developed 8- to 128-fold rises in H-I antibody for the homologous virus while CA H-I antibody levels remained unchanged. Antigenically the CA viruses were unrelated to Sendai or Newcastle virus.

As shown in Table XIV CA virus is distinct antigenically from mumps virus.

Thus, a monkey which developed a 128-fold homologous C-F antibody rise following immunization with CA virus, failed to exhibit a rise for the viral or soluble C-F antigens of mumps. Mumps hyperimmune guinea pig sera, with high homologous C-F antibody levels, failed to fix complement with the CA C-F antigen.

However, the existence of a common antigen was suggested when 2 of 5 mumps patients exhibited a fourfold rise in C-F antibody for CA virus and 4 of

TABLE XIV

Relationship of CA Virus to Mumps Virus

Serum	Reciprocal of antibody titer with indicated virus				
	CA virus		Mumps virus		
	H-I*	C-F	C-F		H-I
			Viral	Soluble	
CA					
Monkey					
Pre-immunization	<5	<4	8	<8	
3 wks. post "	320	256	8	<8	
Mumps					
Human					
Patient 1 pre infection	20	<4	4		
post "	20	8	128		
2 pre "	10	8	<4		
post "	40	8	256		
3 pre "	20	4	<4		
post "	20	4	64		
4 pre "	5	<4	<4		
post "	<5	<4	256		
5 pre "	10	<4	<4		
post "	20	8	256		
6 acute	80				<8
conval.	320				2048
7 acute	5				<8
conval.	20				2048
8 acute	5				<8
conval.	80				1024
Guinea pig					
Pool 1	20	<4	64 or >		
2	80	<4	64 or >	64 or >	
3	<5		64 or >	8	
4	10		<8	64 or >	
Influenza A					
6 pools of guinea pig sera, FM-1, L1049, WS, FW-1-50, L347, and Phila. 51.	<5				
Swine 15 guinea pig pool	5				
Normal guinea pig					
Pool	<5		<8		
Individual guinea pig No. 1 to 4	<5				

* Highest original dilution of RDE-treated serum which produced complete or almost complete inhibition of 4 units of hemagglutinin.

Mumps sera (except for patients 6, 7, and 8) and mumps C-F antibody results kindly supplied by Drs. Gertrude and Werner Henle. Sera from mumps patients 6, 7, and 8 and mumps H-I antibody results supplied through the kindness of Dr. Keith Jensen.

8 patients developed a fourfold or greater H-I antibody rise for CA hemagglutinin. In addition, 3 of 4 pools of mumps hyperimmune guinea pig sera inhibited CA hemagglutinin while 1 pool of normal guinea pig sera, 4 individual guinea pig sera, and 7 pools of influenza A guinea pig antisera did not. In view of the suggested antigenic relationship it is of interest that the possession of significant levels of CA H-I antibody (mumps patients 1, 2, 3, 5 and 6) or C-F antibody (mumps patient 2) did not protect against naturally acquired mumps infection.

Other Viruses.—There was no evidence of any antigenic relationship with the RI-APC group of viruses (Table XIII). Other viruses which were not related antigenically either by neutralization or hemagglutination-inhibition included herpes simplex, the non-APC cytopathogenic agent recovered by Berge from cases of respiratory disease (5), the simian viruses of Hull—SV_1, SV_2, SV_4, SV_5, SV_6, SV_{11}, SV_{12}, and SV_{15} (6), distemper, and the 2 distinct agents recovered from chimpanzees with rhinitis by Sabin (7) and by Morris and Smadel (8).

DISCUSSION

The apparently high incidence of CA virus infection in infants with croup during the first 2 months of this study—5 out of 7—suggests that this virus may be at least one of the etiologic agents of this clinical syndrome but extensive control studies will be necessary to establish a specific etiologic association. The role of the CA virus in other forms of human respiratory disease also awaits the results of future studies. The presence of pneumonitis in 2 and possibly 3 of the 5 infants infected with CA virus may be significant in this regard.

The properties of CA virus place it in the myxovirus group, which except for mumps produces inflammation involving particularly the respiratory system. In addition to the common properties of the myxovirus group described by Andrewes, Bang, and Burnet (14) CA virus shares with naturally occurring strains of influenza A′, B, and C (15, 16), mumps (17, 18), and Newcastle (12) viruses the ability to multiply and produce a cytopathogenic effect in monkey kidney epithelial culture. Of these viruses mumps produces a cytopathogenic change which most closely resembles that seen with CA virus. In both instances the first effect noted is the formation of a local syncytial area; however, the progression to large sponge-like masses containing numerous small vacuoles easily differentiates the CA virus from mumps. Influenza viruses produce predominantly a round cell degeneration in monkey kidney culture (15, 16), while NDV virus initiates primarily a granular degeneration of the affected kidney cells (12). Although CA virus was found to be distinct antigenically from the established members of the myxovirus group, it is of interest that an antigenic relationship with mumps virus was suggested.

In addition to the differences in cytopathogenic effect in monkey kidney

culture, CA virus differs from influenza and Newcastle viruses by its slow growth in the amniotic cavity of the embryonated egg and from mumps virus, which also propagates slowly in the amniotic cavity, by its low level of multiplication. Indeed multiplication of CA virus in the amniotic cavity could be detected only by titration of amniotic fluids in monkey kidney cultures since the quantity of virus produced was insufficient to agglutinate fowl erythrocytes. Since 9 to 10 day old eggs were employed in the CA virus studies and not 7 to 8 day old eggs, which are optimum for mumps virus propagation, the observed differences between CA and mumps viruses must be interpreted with caution.

CA virus differs most strikingly from other myxoviruses in the nature of its reaction with fowl erythrocytes. This interaction has the characteristics of *both* a reversible equilibrium and an enzyme-receptor substrate reaction. These actions may be visualized as the first and second steps in the over-all reaction pattern. The direction of the reversible reaction is predominately toward formation of virus-receptor complex at 4°C. while at 37°C. the equilibrium reaction proceeds mainly toward dissociation. The latter may signify that the thermal energy of the virus particles at 37°C. exceeds the weak binding force of the virus-receptor complex. Under conditions which favor the irreversible enzymatic action, *i.e.* 37°C., only a small fraction of the virus particles are in the combined state and thus receptor destruction proceeds at a slow rate and is overshadowed by reversible dissociation. However, given sufficient virus and time of incubation at 37°C. limited receptor destruction can occur. Experiments supporting the assumption that the initial reaction between certain myxoviruses and red cells is of a reversible nature have been reported by Magill (19) and Tamm (20). However, only in the latter instance with Lee virus and cat erythrocytes was a reversible equilibrium reaction demonstrable and predominant over the irreversible enzymatic action.

SUMMARY

Viruses producing an unusual "sponge-like" cytopathogenic change in monkey kidney tissue culture were isolated from the pharyngeal swabs of 2 of 12 infants with croup. The infants from whom the viruses were isolated and 3 additional patients developed significant increases in neutralizing or hemagglutination-inhibition and complement-fixing or all 3 varieties of antibody during convalescence. The isolated agents appeared to be similar antigenically.

Fluid from infected monkey kidney tissue culture agglutinated chick erythrocytes and in lower titer human "O" red cells. Hemagglutination occurred best at 4°C. and pH 8.0. Agglutination was reversed at 37°C. but resuspension and sedimentation of red cells at 4°C. resulted in a restitution of positive patterns. In addition, the virus was capable of partially removing receptors from the erythrocyte surface, but only when large quantities of virus were incubated with red cells for 24 hours at 37°C. and small doses of hemagglutinin used to test the

treated cells. RDE removed both the erythrocyte receptors and the inhibitor for hemagglutinin present in certain normal sera. Low level multiplication occurred at a slow rate in the amniotic cavity of the fertile hen's egg. Gradocol membrane filtration yielded a size of 90 to 135 mμ. The virus was stable at −70°C. but infectivity was lost after treatment with 20 per cent ether for 15 hours.

The properties of the isolated viruses were consistent with those required for classification in the myxovirus group. No antigenic relationship with influenza A, A′, B, and C, Newcastle or Sendai viruses was found. The viruses were distinct from mumps virus but the existence of a common antigen was suggested.

The high incidence of infection with this new virus in one group of croup patients suggests that it may be at least one of the etiologic agents of this clinical syndrome, but more extensive control studies will be necessary to establish a specific etiologic association. For the present the group will be referred to as CA viruses; *i.e.*, croup-associated viruses.

I wish to thank Dr. Albert B. Sabin to whom I am indebted for much helpful advice and criticism throughout this work.

Addendum.—Since this manuscript was submitted for publication, Dr. A. J. Beale of Toronto was kind enough to send to Cincinnati a cytopathogenic agent representative of the 10 strains recovered from 15 infants with croup during the Winter of 1955–1956. Although the antigenic analysis of the strains is not yet complete, it has been found that they all produce the same cytopathogenic effect in HeLa and human amnion cultures. In our laboratory the Toronto virus (Manganero strain) produced a "sponge-like" cytopathogenic change in monkey kidney culture indistinguishable from that seen with CA virus. The Toronto virus agglutinated chick erythrocytes and the reaction was readily reversible at 37°C.; positive patterns were restored after resuspension and sedimentation at 4°C. Antigenically the Toronto agent could not be distinguished from the Greer strain of CA virus by hemagglutination-inhibition tests with specific monkey antisera prepared against the latter virus.

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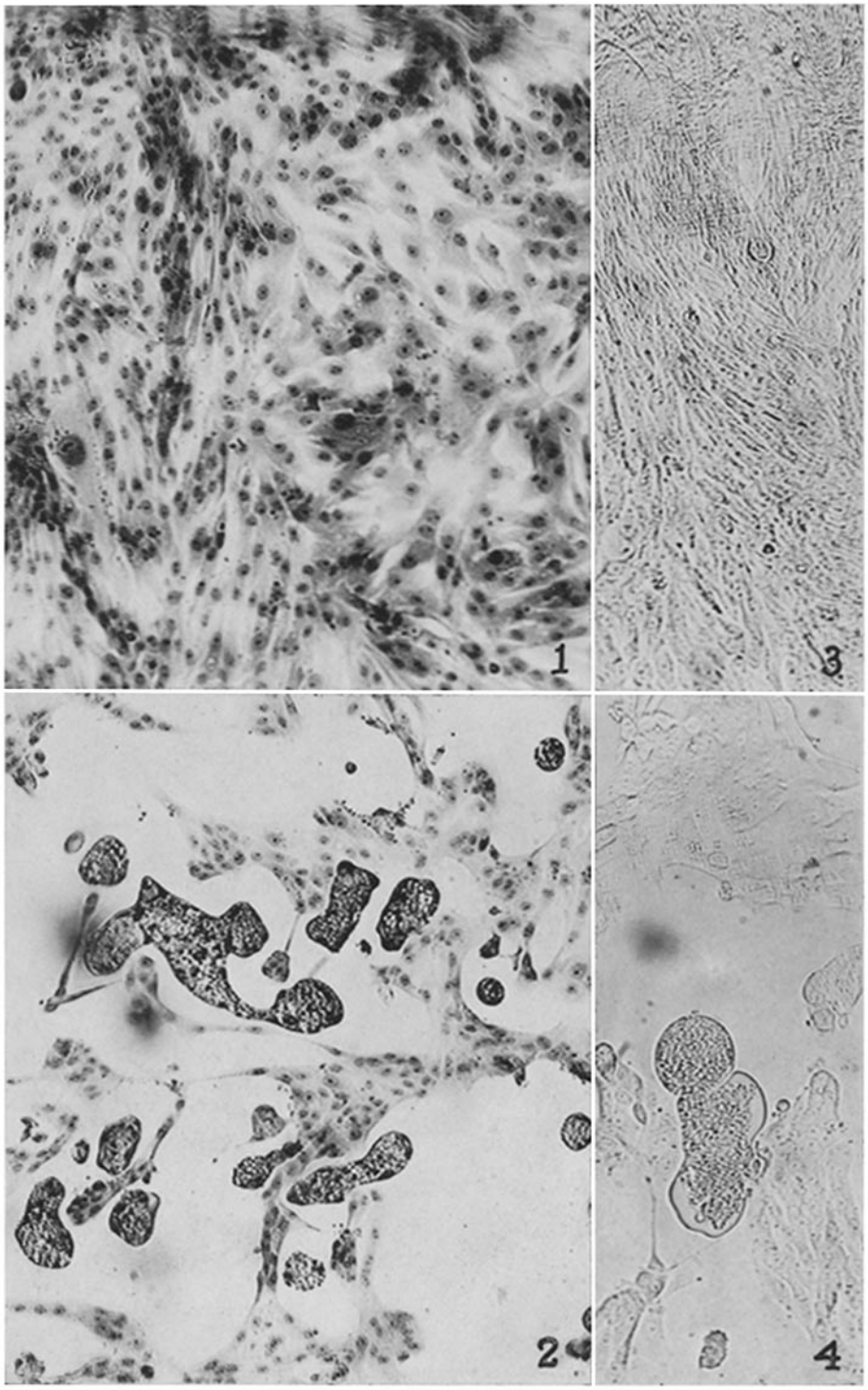
EXPLANATION OF PLATE 47

FIG. 1. Normal monkey kidney epithelium. Stained with hematoxylin and eosin. × 120.

FIG. 2. Cytopathogenic effect produced by "Greer" strain of CA virus in monkey kidney epithelium. Stained with hematoxylin and eosin. × 120.

FIG. 3. Normal monkey kidney epithelium. Unstained. × 120.

FIG. 4. Cytopathogenic effect produced by "Greer" strain of CA virus in monkey kidney epithelium. Unstained. × 120.



(Chanock: Cytopathogenic myxovirus with infantile croup)