Recovery of Cytopathogenic Agent from Chimpanzees with Coryza. (22538)

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During October, 1955, a respiratory illness characterized by coughing, sneezing and mucopurulent nasal discharge occurred in a colony of 20 "normal" chimpanzees at the Walter Reed Army Institute of Research. The present paper describes the isolation of a virus of apparent etiologic significance in the epizootic, establishes an etiologic association between the chimpanzee coryza agent and respiratory illness in a laboratory worker and finally, presents serologic data suggesting that a number of human beings have experienced infection with the chimpanzee coryza virus or an agent closely related to it.

Materials and methods. Chimpanzees and collection of specimens. The chimpanzees in the epizootic were 15 to 20 months old and were obtained from a commercial breeder in Dania, Fla., 3 to 24 weeks prior to their illness. They were housed at the Forest Glen Annex of the Walter Reed Army Institute of Research (WRAIR). Blood specimens for serological study were obtained from individual chimpanzees at outset of the epizootic on Oct. 13, 1955, when 5 of the 20 animals were suffering from clinical coryza, and periodically thereafter until Apr. 18, 1956. Throat swabs were obtained from all animals in the colony on Oct. 17, 1955 when 14 of the 20 animals were clinically ill with coryza; the swabs provided the material employed for viral isolation studies. Another group of somewhat older chimpanzees was used in studying experimental transmissibility of the coryza. The 6 animals in this group had been inoculated previously with material presumed to contain the virus of human infectious hepatitis; they were housed in a different location from the others and had had no direct contact with other chimpanzees for over a year. *Tissue cultures.* Cultures of epithelial-like cells derived from human liver (Chang strain) were prepared by the method of Chang(1). The cultures were grown in roller tubes (1.5 x 13 cm) and stationary bottles (4 x 4 x 14 cm) in nutrient medium consisting of 8 parts Eagle's basal medium(2), 2 parts inactivated horse serum, and 0.2 part L-glutamine. Penicillin (100 units/ml) and streptomycin (20 μg/ml) were added to control adventitious bacterial contaminants. Tubes and bottles contained 1 ml and 15 ml of nutrient fluid, respectively. The cells were fed on the 3rd or 4th day by replacing the old nutrient fluid with an equal amount of fresh nutrient. Cultures were incubated at 36°C and at the time of use were usually 4 to 6 days old. *Isolation of coryza agent.* A fresh (within the hour of collection) throat swab from a chimpanzee (Sue) involved in the epizootic was washed in 2 ml of tissue culture nutrient fluid containing antibiotics. After centrifugation at 3000 rpm for 15 minutes to remove large particles, 0.2 ml of the supernatant was inoculated into a roller tube culture of 4-day-old Chang liver cells. After 4 days incubation the original cell nutrient was replaced with fresh nutrient. Four days later cellular degeneration characterized by rounding, granulation, and sloughing from the tube wall was noted. Serial transmission of the cytopathogenic agent to other tube- or bottle-cultures of Chang liver cells was readily accomplished by passage of suspensions of degenerated cells in their infected fluids. Similar isolation attempts which were made with materials obtained on October 17th from 13 other ill chimpanzees gave negative results. *Serologic procedures. Virus.* Seed virus was obtained by inoculating bottles of liver cells with the chimpanzee coryza agent (CCA) and harvesting cells and fluids 8 days later when the infected cells characteristically showed complete degeneration. After grinding in a TenBroeck grinder the mixture was clarified by centrifugation at 3000 rpm for 15 minutes. The resulting supernatant constituted the seed virus. Infectivity was preserved by storage at –70° in sealed glass ampoules. *Neutralization tests.* Serial 2-fold

dilutions of serum which had been inactivated at 56° for 30 minutes (0.15 ml) were mixed with a constant amount of virus (100 to 1000 tissue culture LD_{50}) contained in 0.15 ml of infected tissue culture material. The mixtures were incubated in a water bath at 37°C for 1 hour after which 0.1 ml of each mixture was added to each of 2 tubes containing normal liver cells. The cultures were examined microscopically for cellular degeneration after a 6- to 8-day incubation period. The neutralization titer was considered the highest dilution of serum completely inhibiting cellular degeneration. Appropriate cell and serum controls and a virus titration were included in each test. *Complement fixation tests.* Satisfactory complement fixing antigen was prepared from infected liver cells grown in medium containing 20% inactivated horse serum. When the horse serum component of the medium was not heated at 56° for ½ hour the material was anticomplementary if used in the complement fixation (CF) procedure employing overnight fixation in the cold, in accordance with the standard technic of the Department of Virus Diseases, WRAIR (3) which was used in the current studies. For use in CF tests human and chimpanzee sera were inactivated for 30 minutes at 56°C and 60°C, respectively. The serum titer was expressed as the reciprocal of the highest dilution giving 75% or greater fixation of complement after overnight incubation at 4°C in the presence of 2 units of antigen and 2 full units of complement. Controls included in each test were antigen (prepared from uninfected liver cell cultures propagated in inactivated horse serum), positive serum (obtained from a man who experienced a laboratory infection, patient B1 in Text Fig. 1, and saline.

Results. Behavior of chimpanzee coryza agent (CCA) in liver cell culture. Inoculation of CCA obtained from the culture of the throat swab of chimpanzee Sue into liver cell cultures produced little or no change during the first 5 or 6 days. On about the 7th day scattered islands of round and granular cells appeared and a few cells were disintegrated and dislodged from the glass wall of the container. Once begun, the process of degeneration spread rapidly and within 24 hours practically all cells were dead and some were floating in the nutrient fluid. Intranuclear and intracytoplasmic inclusions which are eosinophilic in Giemsa-stained cell preparations, were observed in cultures of liver cells infected with CCA. However, similar inclusion-like bodies were demonstrated in uninoculated cells, grown in inactivated horse serum. At the present time the significance of the inclusion-like structures found in infected and uninoculated cells cannot be stated with certainty.

Pathogenicity of CCA for laboratory hosts. Tissue culture materials containing 100 to 10,000 TC LD_{50} of CCA were inoculated by the intracerebral and intraperitoneal routes into one-day-old mice, weanling hamsters and young adult rabbits and guinea pigs. Other 8 to 10 gram mice, young adult rats and 16 to 20 lb chimpanzees were inoculated intranasally. Groups of chick embryos (7 to 11 days old) were inoculated on the chorioallantoic membrane and into the amnionic, allantoic and yolk sacs. With the exception of a single guinea pig that developed persistent fever beginning on the 3rd day, none of the inoculated animals or embryonated eggs other than chimpanzees developed signs of disease during observation periods ranging up to 28 days. The etiology of fever in the guinea pig was ultimately traced to a bacterial infection. Further, the fluids obtained from chick embryos inoculated by various routes failed to agglutinate chicken and human "O" erythrocytes. Tube cultures of human cells derived from conjunctiva (Chang), intestine (Henle) and human embryo fibroblasts (obtained from Microbiological Associates) were found to be less susceptible to the cytopathogenic effect of CCA than liver cells; these cells showed only incomplete degeneration after 16 days incubation. Monkey kidney cells underwent complete degeneration 8 days after infection with CCA but the cytopathogenic effect obtained was sometimes difficult to interpret because of occasional presence in the cultures of adventitious simian viruses(4).

Relation of CCA to epizootic coryza in

TABLE I. Serological Findings with Chimpanzee Coryza Agent (CCA) and Selected Chimpanzee Sera.

					Antibody titers against					
			Chimpanzee coryza agent		Other agents	Influenza (HAI)				
Chimpanzee	Serum date	DD	CF	Neut	RI-67 (CF)	PR-8	FWI-50	FLWI-52	LEE	IB1
Sue (source of CCA)	10/13/55	-3	0*	0*	10	80	20	10	80	80
	12/ 5	50	80	10	10	40	20	10	80	80
Pug	10/13	3	0	0	10	40	20	10	20	80
	12/ 5	53	40	10	10	40	40	10	10	40

* No fixation or neutralization at 1:10 dilution of serum.

chimpanzees at Forest Glen. CCA was found to be related to the epizootic disease of chimpanzees at the Forest Glen Annex of the WRAIR by the use of serologic technics. Illustrative results of CF and neutralization tests performed on sera from 2 of the chimpanzees in the Forest Glen epizootic are shown in Table I. It is seen that in both animals CF antibody against CCA was undetectable in the early sera but titered 1:40 or 1:80 in the sera taken approximately 2 months later. During the same period there was no significant change in CF antibody titer against the RI-APC-ARD virus or in HAI antibodies for any of 5 strains of influenza virus. Finally, neutralizing antibody against CCA developed in both chimpanzees during the period between bleedings. The etiologic relation between CCA and the epizootic disease in the chimpanzees is supported further by the data obtained when sera of all 20 animals involved in the Forest Glen epizootic were examined for specific complement fixing antibody. As shown in Table II all 14 chimpanzees that experienced clinical coryza during the 3rd week of Oct., 1955, subsequently developed specific antibody. Four other animals that did not suffer clinical coryza likewise produced antibody, hence, they presumably experienced unrecognized infection. The remaining 2 animals apparently escaped infection; they neither suffered clinical disease nor developed CCA CF antibody.

TABLE II. CF Antibody Titers Obtained in Sera of Chimpanzees Involved in Coryza Epizootic.

Clinical coryza	Date serum collected	No. of chimpanzees showing antibody titer of <10	10	20	40	80
Yes (14 animals)	10/13/55	13		1		
	12/19		2	6	5	1
	1/24/56		4	5	4	
	4/18	7	3	4		
No (6 animals)	10/13/55	6				
	12/19	3	3			
	1/24/56	5		1		
	4/18	6				

Experimentally induced coryza in chimpanzees. Three chimpanzees, 20 to 24 months of age and weighing 16 to 20 lbs were inoculated intranasally on Feb. 2, 1956 with 1.0 ml of 11th passage tissue culture material containing 10,000 TC ID_{50} of CCA. At the same time 3 other chimpanzees housed in the same room were injected intranasally with an uninfected Chang liver cell preparation. Results of this experiment are presented graphically in Fig. 1. Three days (Feb. 5) after inoculation 2 of the 3 chimpanzees receiving CCA developed respiratory illnesses characterized by sneezing, coughing and subsequently mucopurulent nasal discharge. These signs increased somewhat in severity and persisted at this level for 4 to 5 days; however, the animals were not febrile. By the 14th day the affected chimpanzees were free of signs of respiratory disease. The third chimpanzee in this group (Babe in Fig. 1) remained well throughout the period of observation. Of particular interest is the finding that this animal possessed CF and neutralizing antibodies (titers 1:40 and 1:20, respectively) at the time of inoculation. Two of 3 control chimpanzees that were housed with animals inoculated with CCA also developed disease. Onset of illness in these control animals occurred on the 7th (Feb. 9) and 9th (Feb. 11) day after receiving the non-infec-

Inoculum (2/2/56)	Host	Date	February 5	7	9	11	13	15	17	19	21	23	25	27	March 6
	Chimp														
CCA, 10,000 TC LD_{50} intranasally	Clark	Clinical disease	—	—	—	—	—	—							
		CP agent recovered	+				0								
		Antibodies CF 0	0						160						160
		Neut. 0	0						20						20
	Frank	Clinical disease	—	—	—	—	—	—							
		CP agent recovered	+				0								
		Antibodies CF 0	0						160						160
		Neut. 0	0						80						80
	Babe	Clinical disease													
		CP agent recovered													
		Antibodies CF 40	40						80						80
		Neut. 20							20						10
Normal TC material (contact with chimps experimentally infected with CCA)	Beanie	Clinical disease			—	—	—	—							
		CP agent recovered	+				0								
		Antibodies CF 0	0						160						160
		Neut. 0	0						20						20
	Betsy	Clinical disease				—	—	—							
		CP agent recovered	+				0								
		Antibodies CF 0	0						0					20	20
		Neut. 0	0						10						20
	Blondy	Clinical disease													
		CP agent recovered						+							
		Antibodies CF 0	0						0						20
		Neut. 0	0						10						10
None (contact with chimps experimentally infected with CCA)	Patient B1	Clinical disease					—	—	—	—					
		CP agent recovered							0						
		Antibodies CF	0								80				80
		Neut.	0								40				40

FIG. 1. Experimentally induced coryza in chimpanzees and laboratory infection in chimpanzees and man. 0 = No fixation or neutralization at 1:10 dilution of serum, the lowest dilution tested.

tious cell cultures, or 4 and 6 days, respectively, after the test animals had first exhibited symptoms. None of these chimpanzees developed fever.

From each of the 4 chimpanzees that developed obvious respiratory illness, *i.e.*, 2 test and 2 control animals, an agent cytopathogenic for liver cells was recovered from throat swabs taken on Feb. 8. In addition, a cytopathogenic agent was recovered on Feb. 15 from throat materials from the third chimpanzee receiving non-infectious materials. This animal, (Blondy in Fig. 1) did not show recognizable respiratory disease; nevertheless she developed complement fixing and neutralizing antibodies in minimum amounts. Each of the 5 recovered agents was shown to be similar to or identical with CCA in neutralization tests with specific antisera prepared in rabbits against the Sue strain and in complement fixation tests with a human serum that was known to react with CCA antigen.

It may be mentioned here that the 6 chimpanzees in this experiment (Fig. 1) were challenged by the intranasal instillation of 1 ml of tissue culture material containing 1000 TC LD_{50} of CCA on March 28, 1956, 55 days after the original exposure when each animal possessed demonstrable CF (range 1:20 to 1:160) and neutralizing (range 1:10 to 1:20) antibodies. All 6 chimpanzees remained free of clinical evidence of disease during an observation period of more than a month; moreover, during the same 30-day period there was no appreciable change in serum antibody titers.

Infection of laboratory worker with CCA. During the second week of February 1956, an illness diagnosed clinically as "upper respiratory infection" occurred in a laboratory worker who was working with CCA and who had had intimate contact with the experimentally infected chimpanzees. His illness was characterized by several days of nasal

TABLE III. CCA Complement Fixing Antibody in Persons of Different Ages.

Age groups (yr)	No. sera Tested	Positive	Reciprocal of CF titer
½– 2*	12	1	40
3– 6*	12	0	
7– 9*	9	0	
10–14*	12	2	20, 10
15–18*	13	3	20 for all 3
18†	40	8	80, 40, 20, remainder 10

* Sera from patients with non-respiratory illnesses submitted for diagnostic studies to WRAIR.
† Enlisted personnel, WRAMC, Mar. 1956.

snuffiness, rhinorrhea, cough, malaise, followed by several days of low grade fever and frontal headache. CF and neutralizing antibodies against CCA were undetectable in the sera of the patient taken on Feb. 8, 1956, but titered 1:80 on Feb. 22, 1956. The single attempt to recover a cytopathogenic agent from throat washings taken on the 6th day of this man's illness was not successful. The serologic findings which are shown graphically in Fig. 1 (Patient B1) are taken as presumptive evidence that the CCA was of etiologic significance in the patient's illness.

Serologic reaction of CCA with sera obtained from animals immunized against different viruses. Sera obtained from animals immunized against a variety of viruses were examined in complement fixation and neutralizing tests for their ability to react with CCA. The antisera included those prepared in monkeys against the Enders strain of measles virus*(5), Chanock's croup virus*(6) and Sabin's chimpanzee rhinitis 1954 virus*(7); in rabbits against several strains of Coxsackie virus, (Group A, type 9 and Group B, types 1, 2, 3 and 4), certain "orphan" viruses(8) [Walter Reed prototypes 7043 (untyped), 7045 (ECHO type 6) and 7054 (ECHO type 2)] and simian virus SV_5(4) and in chickens against simian virus SV_{59}(4).) All these antisera failed to react with CCA in complement fixation or neutralization tests.

Occurrence of antibody against CCA in human sera. Results of CF tests to determine the occurrence of CCA antibody in different age groups in the human population are given in Table III. The sera were obtained from patients at the Walter Reed Army Medical Center with a variety of illnesses. It is evident from the tabular data that a number of human beings possessed CF antibodies that react with CCA antigen. Furthermore, such antibodies were uncommon in children but were present in about 20% of the persons in the small group of adolescents and young adults examined. It is of some interest that the 40 young adults listed in the table were barrack mates of patient B1. Paired sera from groups of patients with common cold, bronchitis, cold agglutinin positive primary atypical pneumonia, and RI-APC-ARD infection (3 pairs in each category) were tested for complement fixing antibody against CCA. Certain of these tests were performed by Dr. Sidney Katz in Dr. John Dingle's laboratory in Cleveland using their sera and antigen supplied by us. None of the patients displayed a significant increase in CCA antibody. Nevertheless, certain of the patients possessed throughout their illnesses constant amounts of CCA antibody with titers ranging up to 1:80.

Summary. A virus was recovered from throat materials of a chimpanzee with coryza during an epizootic of respiratory disease in a colony of these animals. The new agent produced degenerative changes in tissue culture, but was not pathogenic for common laboratory animals. The donor chimpanzee as well as other chimpanzees involved in the epizootic developed specific antibodies against the coryza agent during the months following the outbreak. Susceptible chimpanzees following intranasal instillation of tissue culture materials infected with the coryza agent developed clinical coryza and subsequently made specific antibody. A presumptive etiologic association was established between the new agent and respiratory illness in a laboratory worker, but has not been implicated in the illnesses of small groups of patients with several common types of respiratory disease. However, a number of human beings, particu-

* We are indebted to Drs. John F. Enders, R. M. Chanock and A. B. Sabin for these sera.

larly adolescents and young adults, have antibodies in their sera directed against the coryza agent suggesting that these individuals have experienced infection with the new agent or one closely related to it.

1. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

2. Eagle, H., *J. Exp. Med.*, 1955, v102, 37.

3. Department of the Army and the Air Force: Methods for Medical Laboratory Technicians (TM 8-227; AFM 160-14), Rickettsiae and Viruses, Washington, D. C., U. S. Government Printing Office, 1951, 523.

4. Hull, R. N., Minner, J. R., and Smith, J. W., *Am. J. Hyg.*, 1956, v63, 204.

5. Enders, J. F., and Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.

6. Chanock, R. M., Conf. on Viruses in Search of Disease, N. Y. Acad. Sciences, May, 1956, in press.

7. Sabin, A. B., *ibid.*

8. Meyer, H., Rogers, N., Miesse, M., and Crawford, I., *ibid.*

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Long-Term Radiation of Bone Following Administration of C^{14}-Bicarbonate.* (22539)

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Studies on distribution, turnover, and long-term retention of C^{14} (from $C^{14}O_3$) in animal tissues have indicated that from the standpoint of C^{14} radiation hazard, the bone is perhaps of greatest concern(1-5). For several months after injection of 18 μc of $NaHC^{14}O_3$ (a 50 mc man-equivalent), the radiation doses in "active" areas of long bones of mice were greater than the "tolerated dose limit" of 0.05 roentgen equivalent physical (rep) per day. Although it is apparent that one cannot extrapolate directly from animal results to man without some quantitative results on the latter, it seems important to obtain rather extensive data with regard to long-term retention of C^{14} in bones of animals and to estimate radiation being received by the most active areas of such bones. Such data might be of value in rough approximation of the hazard involved in use of C^{14}, and with availability of some human data correlations could be made.

The present paper is a brief report extending and confirming earlier reports from this laboratory(2,3) on long-term retention of C^{14} from bicarbonate by long bones of mice and appropriate radiation calculations.

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Experimental. Each of a group of adult CFW strain mice (3 months of age) was administered intraperitoneally 100 μc of C^{14}-bicarbonate. These animals were sacrificed as indicated in Table I. A femur and humerus were taken from each animal for oxidation and activity assay in a gas phase Geiger counter(6) which had been calibrated against a Bureau of Standards $BaC^{14}O_3$ standard and shown to give results extremely close to the absolute C^{14} content. Corresponding bones from the same animals were fixed in alcohol, embedded in plastic, and ground to provide the desired cross section, and subsequently autoradiogrammed on No-Screen X-ray film. The "active" volume of each bone was calculated from the individual autoradiograms. The average length and width of the parallel autoradiogram lines were measured with a filar micrometer and the "active" bone volume calculated using the procedure and assumptions previously described (3). Based on the total activity determinations on corresponding bones of the same mouse, calculations have been made of the total activity per cmm of "active" bone and the degree of radiation (rep) in "active" bone. Results of these efforts are presented in Table I along with previously reported results(3).