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STUDIES OF PARAINFLUENZA VIRUSES.
I. Clinical, Pathological and Virological Observations‡

The association of parainfluenza virus[1] types 1, 2, or 3, and upper respiratory illness in children has been reported by Chanock, *et al.*,[2,3] and subsequently in both children and adults by many others in various parts of the world.[4-11] Generally, the diagnosis has been made either by virus isolation or by serological means.

In the course of attempting virus isolation from 623 patients hospitalized in the Grace-New Haven Community Hospital between August 1960 and July 1961, a total of 43 hemadsorption viruses was isolated in tissue cultures using the technique originally described by Vogel and Shelokov.[12] Paired sera were available from eight patients, and complete serological studies of these are included in this paper. In addition, two strains of virus possessing hemagglutinating properties were isolated in embryonated eggs inoculated with lung tissues obtained at autopsy from infants after sudden, unexpected death. This report presents the pathological and virological findings in the two autopsied cases, and observations on parainfluenzal infections in patients with croup and other respiratory illnesses.

MATERIALS AND METHODS

Throat swabs or washings

These were collected from patients admitted to the hospital with suspected viral illness and were stored at 4° C. in Hank's balanced solution containing 0.5 per cent gelatin and antibiotics. Specimens were inoculated into tissue cultures within 24 hours, if possible. Embryonated eggs were also used, especially when influenza virus infection was suspected. *Acute-phase serum* samples were obtained at the same time

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the throat specimens were collected, and were stored at -20° C. *Convalescent phase sera* were collected four weeks or longer after onset.

Autopsy materials

Sudden, unexpected death occurred in two infants during a period when Influenza A_2 was active in the New Haven area. Various specimens, including lung, brain, liver and kidney, obtained at autopsy were used for virus study. The fresh tissues were ground in Hank's balanced salt solution containing antibiotics, and after centrifugation, the supernatant fluids were tested for the presence of viral agents in rhesus monkey and human kidney cell cultures, and in seven-day old embryonated eggs. The eggs were inoculated by the amniotic route; amniotic and allantoic fluids were harvested seven days after inoculation and tested for hemagglutinin with chick, guinea pig, and human group O erythrocytes.

Tissue cultures

Rhesus monkey and human kidney tissue cultures were maintained in Earle's balanced salt solution containing 0.07 per cent $NaHCO_3$ (pH 6.8-7.0) and 2 per cent ultra-filtered fetal calf serum.

Virus isolations

Each throat swab was inoculated into three human and/or monkey cell cultures in 0.1-0.3 ml. amounts and incubated at 36°C. On the 4th, 7th, and 10th day one tube was tested for the presence of the hemadsorption phenomenon by addition of 0.2 ml. of 0.5 per cent fresh guinea pig red blood cells followed by incubation at 4°C. for 20 minutes as described by Chanock, *et al.*[8] Indentification of the isolates was made either by hemadsorption-inhibition, hemagglutination-inhibition, and/or neutralization tests using type-specific antisera.

Hyperimmune sera

Type specific antisera of parainfluenza 1, 2, and 3 viruses were prepared in guinea pigs. SV_5 and DA virus antisera were prepared in rabbits.

Antibody studies

Paired sera, obtained from eight patients from whom parainfluenza viruses were isolated, were tested for antibody using the hemagglutination-inhibition (HI) method. Guinea pig erythrocytes were used in all hemagglutination tests and settling of cells was allowed to occur at room temperature. The procedure used for HI tests is described in the accompanying paper.[13] Parainfluenza virus types 1, 2 and 3 used for the antibody determinations were isolated from the patients in the present study, whereas the strain of DA virus used was the original one isolated from human blood obtained at autopsy, as described elsewhere.[14]

CLINICAL OBSERVATIONS

Clinical manifestations of parinfluenzal infection, such as have been previously described,[5, 6, 9, 11] were observed in the patients studied. Table 1 lists the clinical syndromes of several representative cases from whom

TABLE 1. CLINICAL OBSERVATIONS, VIRUS ISOLATIONS AND ANTIBODY RESPONSES IN REPRESENTATIVE CASES

Lab. No.	Age (Years)	Sex	Clinical findings	X-ray	Laboratory*	Virus isolation	Serum**	Reciprocal of HI antibody titers with indicated antigen: Para-1	Para-2	Para-3	DA
3512	4½	F	Rhinorrhea, fever, cough	Right middle lobe pneumonia	WBC 23,000	Para-1	A	<10	80	80	<10
							C	80	80	160	40
3515	2	M	Croup, no rales	Increased broncho-vesicular markings	WBC 9,700	Para-1	A	<20	20	20	<20†
							C	>1280	40	10	40
3539	3	F	Croup, T. 100°F.	Normal chest	WBC 7,400	Para-1	A	<10	20	80	<10
							C	20	20	80	<10
3605	20 mo.	F	Croup, T. 100°F.	Normal chest	WBC 18,000	Para-1		NOT DONE			
3614	7	F	Fever—104°F. abdominal pain, cough. Right pleural effusion	Right lower lobe and right middle lobe infiltrate. Right pleural effusion	WBC 21,000 pneumococci cultured from nose and throat	DA	A	<10	20	320	40
							C	10	40	320	>320
3635	7	F	Croup	Normal chest	WBC 7,130	Para-2		NOT DONE			
3662	15 mo.	F	Tachypnea, subcostal retractions, wheezes, and rhonchii	Normal chest	WBC 16,800	Para-2		NOT DONE			
3663	5 mo.	M	Cough, rhonchii both lung fields	Normal chest	WBC 13,000	Para-2	A	<10	<10	<10	<10
							C	<10	<10	20	<10
3665	22 mo.	M	Croup, T. 100.4°F.	Normal chest	WBC 13,000	Para-3	A	<10	<10	<10	<10
							C	80	20	160	20
3668	1 mo.	M	Cough, vomiting, coarse bilateral rales	Patchy bilateral infiltrates	WBC 15,000 pneumococci cultured from nose and throat	Para-2	A	<20	<20	<20	<20
							C	40	80	80	80
3674	6½	M	Croup, T. 102°F.	Normal chest	WBC 9,800	Para-2	A	<10	<10	80	<10
							C	10	160	160	160

*All patients had nose, throat and blood cultures for bacterial pathogens. Normal flora was found in all cases except where indicated.
**A=acute phase serum; C=convalescent phase serum.
†Serum dilution 1:20 was the lowest dilution tested; therefore <20 means considerably less than 20.

viruses were isolated and infections were confirmed serologically. As is well recognized, clinical criteria alone do not suggest the type of etiologic agent; e.g., cases of croup were associated with either parainfluenza virus type 1 (cases 3515, 3539, and 3605), parainfluenza virus type 2 (cases 3635, 3668, and 3674), or parainfluenza virus type 3 (case 3665).

The isolation of a "DA" virus from the throat swab of a seven-year old girl represents the first human isolation of this agent with serological confirmation (Table 1, case 3614). This patient entered the hospital with a fever of 104°F., severe abdominal pain and evidence of right middle lobe and right upper lobe pneumonia with effusion. Pneumococci were cultured from the nose and throat, and leukocytosis of 21,000 was present. The patient was treated with penicillin and improved with a gradual lysis of fever over a six-day period. The presence of another etiological agent, i.e. the pneumococcus, and the clinical picture would appear to cast doubt on the importance of "DA" virus in this patient's disease, but the serological evidence indicates at least a concurrent infection with this agent.

PATHOLOGICAL FINDINGS

Two sudden, unexpected deaths in infants occurred during March 1961, at which time a subgroup of Influenza A_2 virus was prevalent in the New Haven area.* Various tissues, including lung, brain, spleen, kidney and liver, which had been obtained at autopsy, were sent to the Virus Laboratory for culture. Parainfluenza virus type 2 was recovered in the amniotic cavity of embryonated eggs inoculated with lung from both cases. The pathological findings of these two cases are as follows:

Case 3807. Autopsy No. 15539.

A 30-hour old Negro boy had been born after 32 weeks of gestation (birth weight —2490 gr.). The mother's membranes had ruptured 26 hours before delivery, and during the immediate post-partum period the mother's temperature had risen to 102°F. No cultures were obtained, but the child was given penicillin and streptomycin prophylactically. The placenta and amniotic fluid had appeared grossly normal. Twenty-four hours after birth the infant began to suffer apneic spells which continued intermittently to death in spite of treatment with assisted and artificial respiration.

Autopsy was performed 16 hours after death. Externally, no cyanosis or other abnormalities were seen. The cord stump and the surrounding area were not grossly inflamed, but microscopic sections showed polymorphonuclear infiltration with edema. Bacterial cultures were sterile.

* The authors are indebted to Dr. R. Q. Robinson, Communicable Disease Center, International Influenza Center of the Americas, for confirming the identification of the agents.

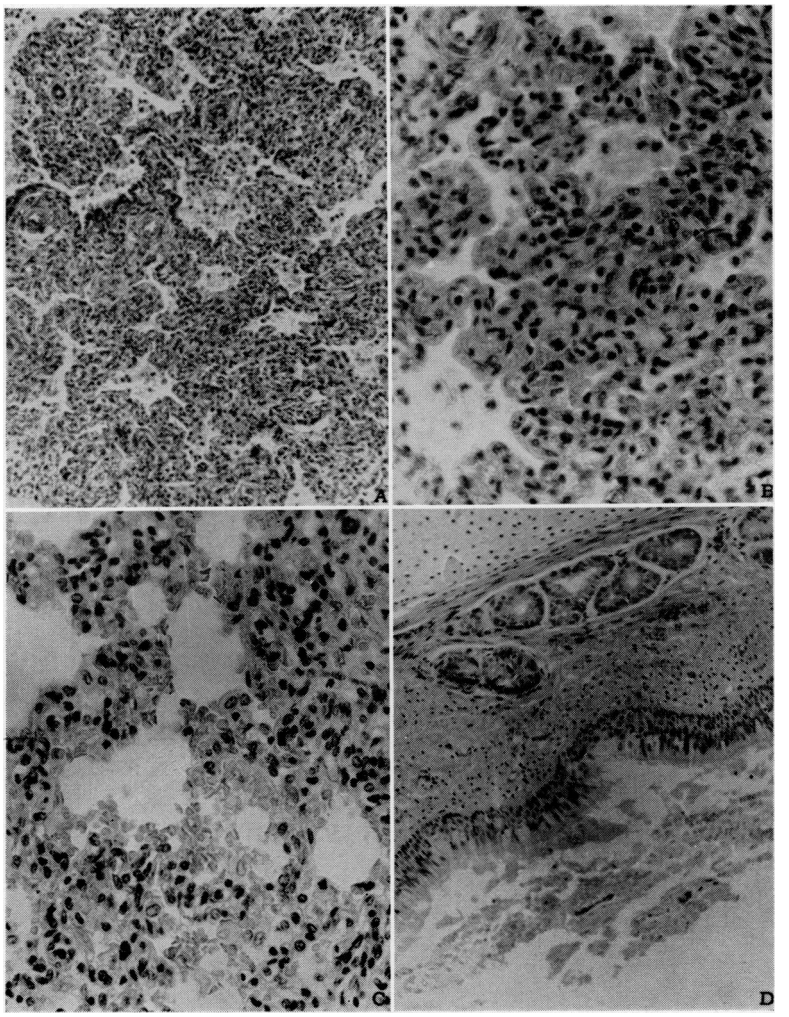

FIGURE 1.

A. Section of lung (Hematoxylin and Eosin x 120) showing marked thickening and infiltration of the interalveolar septa with polymorphonuclear and mononuclear leukocytes. Considerable intra-alveolar edema and leukocytic infiltration are also apparent. Free red blood cells can be seen in some alveoli.
B. Section of lung as in A (x 350).
C. Section of lung showing thickening and extensive infiltration of the interalveolar septa with chiefly monuclear leukocytes. The alveoli contain edema fluid, red blood cells and occasional non-pigmented macrophages. (Hematoxylin and Eosin, x 300).
D. Section of trachea showing proteinaceous exudate and red blood cells (Hematoxylin and Eosin, x 120).

The heart showed great enlargement of both sides. There were small subepicardial petechiae over the anterior portion. The lungs were homogeneously dark red and moderately firm with generalized edema; however, no bronchial occlusions or thick mucus could be detected. Microscopically, bilateral intra-alveolar edema, exudation of polymorphonuclear leukocytes and atelectasis were present as shown in Figure 1, A and B. There was considerable vascular congestion as well as intra-alveolar hemorrhage. No bronchial changes were seen.

The rest of the autopsy, including an examination of the brain, revealed no pathologic changes. Final pathologic diagnosis: Acute omphalitis and bilateral interstitial pneumonia; prematurity; severe atelectasis.

*Case 3808. Autopsy No. A15540.**

A 5-week old white male infant had been spontaneously delivered after a normal, full-term pregnancy, and had been in excellent health all his life. On the morning of his death he was found apneic in bed and was pronounced dead in the Emergency Room. Artificial respiration, oxygen and closed chest cardiac massage had been to no avail. The death had not been preceded by warning symptoms or signs.

Autopsy was performed three hours after death. The body weighed 4920 gr. and had a crown-heel length of 57.5 cm. There was extreme cyanosis. The eyes and nose were normal. A small amount of crusted blood was seen coating the tongue. The umbilicus was well granulated and revealed no evidence of infection. Cerebro-spinal fluid, blood and lung cultures for bacteria were all sterile.

On opening the chest, the pleural and pericardial surfaces were seen to be smooth and glistening, except for a moderate number of petechiae on the anterior visceral surfaces and in the thymus. The heart and great vessels were normal in structure, and there was no miscroscopic evidence of myocarditis or other pathologic change.

The right lung weighed 39 gr., the left lung 35 gr. Externally, raised emphysematous regions were present in all lobes. There was patchy blue-red discoloration at both the apices and bases, as well as several larger hemorrhagic regions. The consistency was spongy, and cut surfaces showed peribronchial hyperemia, but no evidence of aspiration. On microscopic examination, focal, subpleural atelectasis was seen, as well as edema and hemorrhage (Fig. 1-C). The septal walls were thickened and extensively infiltrated with mononuclear cells and occasional polymorphonuclear cells. Many alveoli, especially near the pleura, were filled with proteinaceous fluid, moderate numbers of red blood cells, and distended non-pigmented macrophages. No bacteria or inclusion bodies were seen. Several bronchi contained edema fluid and red cells.

The tracheal mucosa was covered by a thin layer of bloody material in which a few colonies of bacteria were seen. However, there was no cellular infiltrate (Fig. 1-D).

The brain showed no pathologic changes except vascular congestion. The rest of the organs, including the liver and kidneys, revealed no lesions. Final pathologic diagnosis: Focal hemorrhagic interstitial pneumonia; petechiae on heart, lungs and in thymus.

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*Autopsy was performed by Dr. James Luke, Department of Pathology, Yale University School of Medicine.

VIROLOGIC AND SEROLOGIC FINDINGS

Autopsy specimens

Details of the isolation of parainfluenza virus type 2 in the amniotic cavity of embryonated eggs inoculated with lung suspension of cases 3807 and 3808 are shown in Table 2; these viruses were not obtained in any of the tissue cultures inoculated with the same materials. All eggs were

TABLE 2. ISOLATION OF PARAINFLUENZA VIRUS TYPE 2 FROM AUTOPSY MATERIAL

		Virus Isolations							
		Tissue cultures				*Embryonated eggs*			
		Fluid medium			*Agar overlay*			*No. positive*/ No. inoculated*	*Hemagglutin titers in amniotic fluid*
Lab. No.	*Specimens*	*HK*	*Rh*	*Pates*	*Rh*	*No. pass*	*Days old*		
3807	Lung suspension 20% (w/v)	0/6	0/16	0/9	0/3	E_1	8	1/4	N.D.
						E_2	8	4/9	1:5
						E_3	9	1/3	1:10
						E_4	8	2/3	1:20
3808	Lung suspension 20% (w/v)	0/27	0/9	0/9	0/6	E_1	8	1/4	N.D.
						E_2	8	2/6	1:10
						E_3	9	2/3	1:20
						E_4	8	3/6	1:20

*No. of eggs to yield positive hemagglutinin.
N.D.=Not done.

inoculated at 8—9 days of age by the amniotic route, and harvested 5—7 days after inoculation. Amniotic and allantoic fluids were tested for presence of a hemagglutinin using 0.5 per cent guinea pig and chick red blood cells. Hemagglutination was obtained only in amniotic fluid using guinea pig red blood cells and was not observed in any of the allantoic fluids. The hemagglutinin titers were low and no significant increase was evident even after 3—4 egg passages. No virus was obtained from rhesus monkey or human kidney cell cultures inoculated with positive amniotic fluids.

Clinical specimens

During the 12-month period, i.e., between August 1960 and July 1961, 43 hemadsorption viruses were isolated from 623 patients tested. As a

routine, freshly obtained throat swabs from each patient were inoculated into tissue cultures maintained in an acid (pH 6.8) medium containing fetal calf serum. It was found that similar cultures maintained in Earle's salt solution, containing 0.5 per cent lactalbumin hydrolysate and 0.23 per cent $NaHCO_3$ (pH 7.8), were unsatisfactory. Of the 43 isolates, 20 strains lost their viability during storage before identification was made; 11 strains

TABLE 3. SEROLOGICAL CROSS-REACTIONS OF ANTISERA TO PARAINFLUENZA VIRUSES

			Reciprocal of antibody titers to the following virus strains					
	Hyper-immune	*Animal*	*Hemagglutination-inhibition*				*Neutralization***	
*Experiment**	*serum*	*species*	*Para-1*	*Para-2*	*Para-3*	*DA*	*DA*	*3614*
A	Para-1	Guinea pig	320	20	<10	320	80	100
	Para-2	"	<10	320	<10	320	80	80
	Para-3	"	<10	<10	160	40	10	10
	SV_5	Rabbit	<10	<10	<10	640	160	100
B	Para-2	Guinea pig	<10	160	<10	<10	<10	<10
	Para-3	"	<10	<10	640	<10	<10	<10
C	Pre-	Rabbit	<10	<10	<10	<10	<10	<10
	Post (DA)	"	<10	<10	<10	320	320	160

*A. Sera were supplied by a commercial source.
B. Sera were obtained through the courtesy of Dr. R. Q. Robinson of International Influenza Center for the Americas, Communicable Disease Center, Atlanta, Georgia.
C. Antiserum against DA virus was prepared in a rabbit by intravenous inoculation.
** Neutralization test for DA virus was plaque reduction method.
Neutralization test for 3614 virus was hemadsorption-inhibition method.

were identified as parainfluenza virus type 1, nine were parainfluenza type 2, two were parainfluenza type 3, and one strain was identified as DA virus (case 3614). The identification of virus from case 3614 was confusing because its infectivity was neutralized by the type specific antisera of parainfluenza 1 and 2, as well as SV_5, which were obtained commercially (Table 3—last column in Experiment A). On the other hand, case 3614 virus was not inhibited by antisera of parainfluenza virus types 2 or 3 obtained from the Communicable Disease Center (CDC) (Table 3—last

column in Experiment B). Accordingly, these antisera were tested against known laboratory strains of the viruses in question. As shown in Table 3, antisera for parainfluenza 1 and 2 (Experiment A), which were prepared in guinea pigs,* inhibited not only the homologous virus but also the stock DA virus. However, hyperimmune parainfluenza sera obtained from CDC did not show such cross reactivity. Thus, the inhibition of 3614 virus by the type specific antisera of parainfluenza 1, 2 and SV_5 in Experiment A was the result of a common antibody present in these hyperimmune sera. The identity of the 3614 isolate was not established until the specific inhibition of this virus was accomplished with serum obtained from a rabbit immunized with DA virus; the pre-immunization serum obtained from the same rabbit failed to inhibit the agent (Table 3—last column in Experiment C). Subsequently, a significant rise in antibody titer to DA virus was demonstrated in serum from patient 3614 (Table 1), thus confirming the virus identification.

Antibody responses

Complete serological studies of the eight cases from whom viruses were isolated are included in Table 1. Heterotypic antibody rises were apparent in several instances, although homotypic responses were generally higher. Parainfluenza type 1 was isolated from patients 3512 and 3515. These patients had a greater than fourfold rise in HI titer to para-1 virus, but also had a fourfold rise to DA virus and in case 3515, to para-2 virus. Similarly, antibody titer increases were obtained to DA virus in paired sera from case 3674 from whom para-2 virus was isolated. In case 3665, from whom para-3 virus was isolated, an antibody rise to para-1 was obtained in addition to the homotypic response.

DISCUSSION

Croup-associated (CA) virus, now designated as parainfluenza virus type 2, was first isolated from children with acute laryngotracheitis by Chanock.[3] Subsequently, parainfluenza virus type 1 was found to be associated with infectious croup in children in the District of Columbia.[5] Clinical and bacteriologic studies of infectious croup seen on the pediatric service of the New Haven Hospital from 1937 to 1948 were reported by Rabe in 1948, and the clinical entity of "viral" croup was defined.[15] The

*It was found later that most of the normal guinea pig sera contained high titers of DA antibody.[18]

cases in the subsequent 10-year-period were reviewed by Hartmann, and serologic tests for parainfluenza virus types 1 and 3 antibodies in sera from normal New Haven children were reported.[16] In the present study, virus isolations from cases of croup and other respiratory illnesses occurring in the New Haven area have been made, and types 1, 2, and 3 parainfluenza viruses have been recovered.

The hemadsorption technique greatly facilitated recognition of these agents. However, difficulties are often encountered in the isolation of the parainfluenza viruses due to the lability of this group of agents. Generally, fresh specimens are more suitable than frozen samples for recovery of infectious viruses. In addition, the elimination of lactalbumin hydrolysate in the growth medium and the lowering of pH of cell cultures were found to be favorable conditions for the growth of the parainfluenza viruses. Similar results have been reported previously with other myxoviruses.[17, 18]

Isolation of parainfluenza type 2 virus from the autopsy materials was surprising. Influenza A_2 was present in the area at the time of death of the two infants, and egg inoculations were undertaken with the thought that Influenza A_2 virus might be the etiological agent. However, parainfluenza virus type 2 was isolated from lung tissue in both instances, and the pathological picture was compatible with viral pneumonia. These results cannot, however, be interpreted as decisive evidence that parainfluenza virus infection was the major cause of death, although the association of viral infection and sudden death in infants has been reported previously.[19, 20]

The isolation of DA virus with serologic confirmation in case 3614 was of some importance in relation to the host range of this agent. DA virus was originally isolated from human blood obtained at autopsy.[14] Subsequently, it was found that DA and SV_5, a simian parainfluenza virus,[21, 22] were serologically indistinguishable. The question arose whether DA virus was actually of human origin, or whether it was a contaminant of the monkey cell culture system. Virus isolation and identification in addition to an appropriate rise in antibody titer were obtained in case 3614 of the present series; these observations support the view that man may be one of the natural hosts of the DA-SV_5 group of viruses*

* It was proposed by one of the authors (G.D.H.) that the DA-SV_5 group of viruses be designated as parainfluenza virus type 5 at the VIII International Congress for Microbiology, Montreal, 1962.

SUMMARY

A total of 43 strains of parainfluenza viruses, types 1, 2, or 3 were isolated from patients with a variety of respiratory illnesses including croup and pneumonia during the winter season of 1960-1961 in the New Haven area.

"DA" virus was isolated from the throat swab of one case and serologic studies demonstrated a greater than fourfold rise in antibody to the "DA" virus. Parainfluenza virus type 2 was isolated from lung tissues obtained at autopsy in two cases of sudden, unexpected death in infants.

ADDENDUM

After this paper had been submitted for publication, a third isolation of parainfluenza type 2 virus was obtained from the nose swab of a 2-month old baby who died less than an hour after arrival at the Emergency Room. The child had been previously healthy, but was suddenly taken ill and had been brought to the hospital two hours after onset of illness, characterized by fever, vomiting and lethargy. Blood drawn in the Emergency Room had a white cell count of 7,100 with a differential count showing 23 per cent polymorphonuclear leukocytes, 70 per cent lymphocytes and 7 per cent monocytes. Blood and cerebro-spinal fluid cultures for bacteria and viruses were negative but the nose swab collected shortly before death was positive for a hemadsorption virus. Autopsy* findings of note were limited to 1) the lungs, where the changes were interpreted as those of acute focal bronchiolitis and interstitial pneumonia, more compatible with a viral than with a bacterial etiology; and 2) the lymphoid tissue which showed moderate, generalized hyperplasia.

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