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Studies on the Pathogenesis of Parainfluenza Type 3 Virus Infection in Hamsters[1]

By

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With 6 Figures

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The etiological relationship of parainfluenza viruses to human respiratory illnesses has been well established (1–3). Type 3 parainfluenza virus (Para 3) produces a spectrum of clinical illnesses in children including rhinitis, pharyngitis, bronchitis, bronchiolitis and bronchopneumonia, while in adults the infection is usually mild, consisting primarily of coryza. *Craighead* et al. (4) reported the multiplication of Para 3 virus in hamster lungs following intranasal inoculation. However, there has been no systematic study of this infection in experimental animals. The present paper is concerned with the pathogenetic studies of Para 3 infection in hamsters by means of infectivity titrations, immunofluorescent staining and serological response. Effect of corticosteroid administration on the infection is also presented.

Materials and Methods

Virus. The strain of parainfluenza 3 (Para 3) virus was received through the courtesy of Dr. *R. M. Chanock*, National Institutes of Health. The virus has been maintained in monkey kidney tissue cultures in our laboratory. The TCD_{50} of each stock varied between 4.5 and 6.5 logs per 0.1 ml.

Antisera. Hyperimmune sera against the Para 3 virus were prepared by inoculating monkeys with a mixture of virus and mineral oil. The schedule of inoculation and bleeding was similar to that used for preparation of antisera against enteric viruses in this laboratory (5). The neutralization titer of the pooled serum against 100 TCD_{50} was $\geq 1/16{,}000$ and the hemagglutination-

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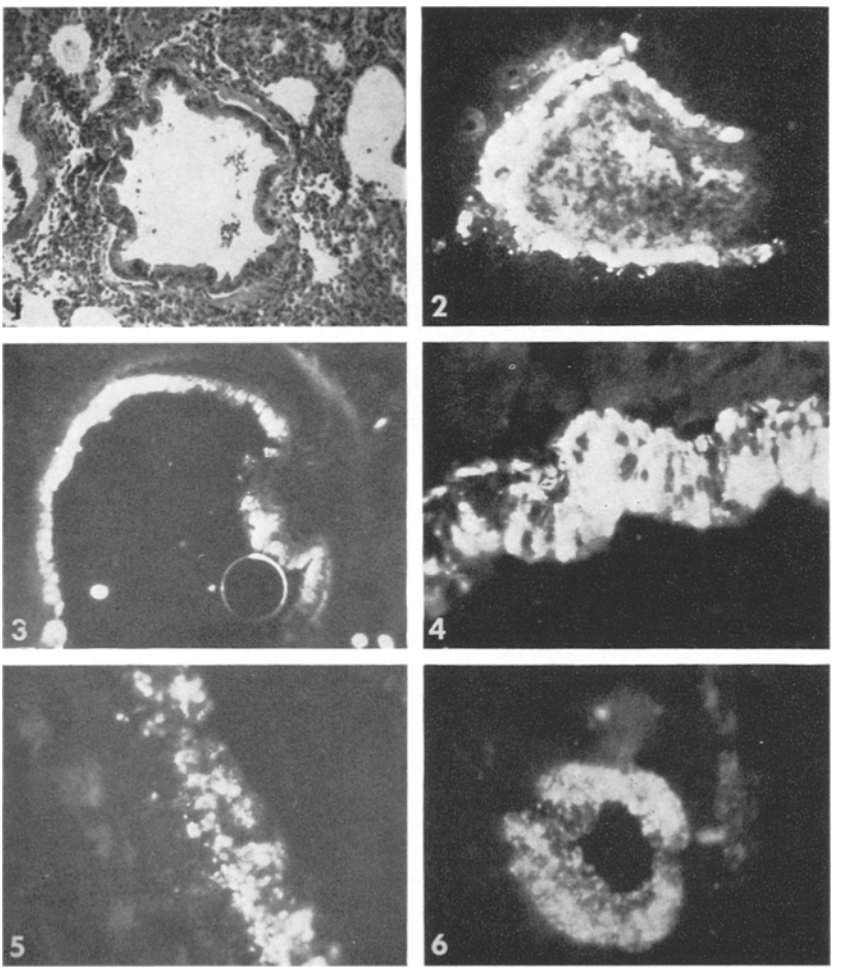

Fig. 1. Paraffin section of hamster lung 3 days after parainfluenza type 3 infection. Normal appearance of ciliated columnar epithelium, a bronchus and cellular elements in pulmonary tissues. Hematoxylin and eosin. ×80.

Figs. 2–6: Frozen sections stained with anti-parainfluenza type 3 fluorescent conjugate. The white areas represent specific fluorescence of viral antigens.

Fig. 2. Nasal turbinate from 4-week-old hamsters 3 days after intranasal inoculation of virus. Intense fluorescence in epithelial cells. ×160.

Fig. 3. Trachea from 4-week-old hamsters 4 days after intranasal inoculation of virus. Viral antigen in ciliated epithelial cells. ×80.

Fig. 4. Bronchus from 4-week-old hamsters 2 days after intranasal inoculation of virus. Viral antigen in cytoplasm of infected epithelial cells. Nuclei were devoid of antigen. ×320.

Fig. 5. Nasal turbinate from 4-week-old hamsters 3 days after intranasal inoculation of virus. Note fluorescent granules of various size in cytoplasm of infected epithelial cells. ×320.

Fig. 6. Bronchus from 3-day-old hamster 3 days after intranasal inoculation of virus. Note viral antigen as granular and homogeneous fluorescence in epithelial cells. ×160.

inhibition titer (HI) was $\geq 1/4096$, using 4 HA units according to standard procedures (6).

Hyperimmune anti-Para 3 sera were also prepared in rabbits by two different methods.

a) Ten ml of undiluted tissue culture fluid containing virus were injected into each rabbit intravenously (i.v.). One week later, another 10 ml of virus were given intraperitoneally (i.p.). Rabbits were bled by cardiac puncture one week after the second injection. With this method, the antisera usually had an HI titer between 1/256 and 1/640.

b) Five ml of undiluted virus in tissue culture fluid were mixed with an equal amount of complete Freund's adjuvant and injected intramuscularly (i.m.) into four different sites of each rabbit. Three weeks later, 5 ml of undiluted virus were given i.p. followed 2 weeks later by 2 ml of the same virus i.v. Rabbits were bled 7—9 days after the last injection. With this method, the antisera had an HI titer of 1/5120.

Preparation of Fluorescent Antibody. The aforementioned hyperimmune Para 3 serum pools were first precipitated with ammonium sulfate and then conjugated with fluorescein isothiocyanate (7). After absorption with mouse liver powder, the labeled antibody solution was ready to be used for direct fluorescent staining. Samples of unlabeled sera were diluted with saline and used for indirect fluorescent staining, with anti-rabbit globulin sheep serum labeled with fluorescein as the fluorescent conjugate (8).

Hamsters. Golden hamsters were obtained from different dealers. For early experiments, hamsters were obtained from a local dealer in Kansas City, Kansas. In subsequent experiments where a large number of animals was required, hamsters were obtained from the Greenfield Village animal farm in St. Joseph, Missouri. Hamsters for each individual experiments were always purchased from a single dealer.

Inoculation of Animals. Hamsters of various ages were used for the experiments. After light ether anesthesia, 0.05 ml (for 3- to 7-day-old hamsters) to 0.1 ml (for 4-week or older hamsters) of 10^{-1} virus suspension representing approximately 10,000 TCD_{50} doses was introduced into each nostril. At varying intervals, hamsters were sacrificed by intraperitoneal injection of 60 mg of sodium pentothal (Abbott). Nasal turbinates, tracheas and lungs and sometimes other organs such as liver, spleen, kidney, etc. were harvested and immediately frozen in a mixture of propyl-alcohol and dry ice. A portion of the same tissues was preserved in 10% formalin for paraffin sections. In baby hamsters, because the organs were too small to be harvested individually, the entire carcass was frozen and sections were cut at levels of nasal turbinates, larynx, lung, liver and intestines.

Sectioning and Staining. Frozen sections of 6 microns thickness were cut in a cryostat as previously described (9). After acetone fixation, slides were stained with fluorescent antibody solution for 30 minutes at room temperature and then mounted with buffered glycerine. For indirect staining, 30 minutes of diluted unlabeled serum followed by 30 minutes of fluorescent anti-rabbit globulin at room temperature were used. Controls for specificity were included as follows:

a) absence of specific fluorescence in hamster tissues not infected with Para 3 virus,

b) absence of specific fluorescence in infected tissue section stained with heterologous fluorescent conjugate,

c) diminution of specific fluorescent staining by inhibition with homologous unlabeled antiserum.

Infectivity Titrations. Two hamsters were sacrificed each time. Each tissue was harvested separately. After frozen sections were cut, the nasal turbinates or tracheal pieces from the two hamsters harvested on the same day were pooled and ground as a 10% suspension in pH 7.0 phosphate-buffered saline. Ten per cent of the infected lungs from individual animals were prepared in the same manner. Serial 10-fold dilution of tissue suspensions were made in buffered saline with 1000 units of penicillin and 1000 micrograms of streptomycin per ml added. Each dilution was inoculated into 4 tubes of monkey kidney tissue cultures and incubated at 37° C for 72 hours. The presence of virus was detected by hemadsorption method with 0.4% of guinea pig erythrocytes (10). TCD_{50} was calculated according to the *Reed* and *Muench* method (11).

Table 1. Infectivity Titrations and Immunofluorescent Staining on Tissues from 4-Week-Old Hamsters Intranasally Infected with Parainfluenza 3 Virus

Days after inoculation	Tissues examined					
	Nasal turbinate		Trachea		Lung	
	TCD_{50}	FA	TCD_{50}	FA	TCD_{50}	FA
1	1.0—4.3[1]	0[2]	<1.0—3.3	0	2.5—4.0	0
2	1.7—4.7	0→++	>1.0—4.7	+++	4.7—6.3	+++
3	2.3—3.5	0→++	3.5—5.7	++	4.5—5.3	+++
4	3.5—5.3	++	4.5—4.8	+++	4.0—5.7	++++
5	3.7	+++	4.7	++++	4.2—5.2	++++
6	1.5	0	2.5	0	<1.0	+
7—14	<1.0	0	<1.0	0	<1.0	0

[1] Log_{10} TCD_{50} per 0.1 gram tissue.

[2] Intensity and extent of viral antigen localization as seen by fluorescent antibody staining. 0 negative; + minimum specific fluorescence; ++++ maximum specific fluorescence.

Experimental Results

Pathogenesis in 3- to 4-week-old Weanling Hamsters

With an intranasal inoculum of approximately 10^4 TCD_{50}, no outwards signs of infections in the inoculated hamsters were seen up to 14 days of observation. During autopsy, gross observation did not reveal any pneumonic changes in the lungs. Conventional histological examinations of paraffin sections stained with hematoxylin and eosin of the respiratory tissues did not show any appreciable pathological changes (Fig. 1). The ciliated columnar epithelium lining the nasal turbinates, the trachea and the bronchi were intact, and no abnormal cellular infiltration was noticeable.

Despite the negative findings in tissues of inoculated hamsters by conventional histopathological methods, fluorescent antibody staining in frozen sections and infectivity titrations of such tissues did demonstrate an active infection had taken place. At 24 hours after inoculation, although there were no detectable viral antigens seen in the respiratory tissues, there was a moderate amount of infectious virus varying from 1.0 log to 4.3 logs/0.1 g of nasal turbinates, trachea or lungs. Since a total of only 10^4 TCD_{50} was given intranasally, allowing a conservative esti-

Table 2. Infectivity Titrations and Immunofluorescent Staining on Tissues from 3-Day-Old Hamsters Intranasally Infected with Parainfluenza 3 Virus

Days after infection	Animal No.	Nose FA[1]	Larynx FA	Trachea FA	Lung	
					TCD_{50}[2]	FA
1	1233	++	+	+	3.3	+
	1234	++	0	+	ND	++++
	1553	0	ND	ND	<1.0	0
	1554	0	ND	0	<1.0	0
2	1235	++	++	+++	4.0	++++
	1236	++++	±	+++	4.2	++++
	1559	0	ND	0	<1.0	0
	1560	0	ND	0	2.7	0
3	1239	+++	+	++++	2.7	++++
	1240	+++	ND	+++	2.7	++++
	1563	+++	++	ND	4.9	+
	1564	0	ND	ND	3.0	0
4	1241	++++	0	++++	3.7	++++
	1242	++++	++	+++	1.7	?
	1567	+++	+	+	5.0	+++

[1] + minimum fluorescence; ++++, maximum fluorescence.
[2] Log_{10} TCD_{50} per 0.1 gram tissue.

mation of a dilution factor of 30 (estimating the total weight of nasal turbinates, trachea and both lungs to be 3.0 grams), the recovery of more than $10^{2.5}$ TCD_{50}/0.1 g of tissue would indicate an active virus multiplication had taken place. Results from several experiments are summarized in Table 1 and Table 4, Group A.

Between the second and the fifth days after inoculation, viral multiplications in the respiratory tissues were more apparent. This was particularly evident in the trachea and lungs, where the TCD_{50} had usually gone above 4.0 logs/0.1 g of tissues. Also definite specific viral antigens were detectable in the ciliated epithelium lining the nasal turbinates, the

trachea and the bronchi of the lungs (Figs. 2, 3, 4). The tracheal and bronchial epithelium was more regularly involved than the epithelium of the turbinates. The viral antigens appeared as brilliant fluorescent granules of varying sizes situated in the cytoplasm of infected cells (Fig. 5).

Table 3. Infectivity Titrations and Immunofluorescent Staining on Tissues from 7-Day-Old Hamsters Intranasally Infected with Parainfluenza 3 Virus

Days after infection	Animal No.	Nose FA[1]	Larynx FA	Trachea FA	Lung	
					TCD_{50}[2]	FA
1	1319	±	0	0	ND	+
	1320	+	0	0	2.0	+
2	1328	+	+	++	4.2	++
	1329	+++	+	+	4.5	+++
3	1330	+++	±	++	2.0	+++
	1331	+++	±	++	3.3	++++
5	1348	++++	ND	+++	ND	++++

[1] + minimum fluorescence; ++++ maximum fluorescence.
[2] Log_{10} TCD_{50} per 0.1 gram tissue.

Table 4. Results on Antibody Titers of Various Anti-Parainfluenza 3 Sera and Their Effect on Indirect Immunofluorescent Staining of Infected Hamster Lungs

Sera from:	Para-3 antibody titer			Indirect FA used at 1/10
	CF	HI	Neut.	
Pooled guinea pig[1]	64[2]	64	64	0
Human Convalescent[1]	64	64	256	0
Human Convalescent[1]	8	8	128	0
Rabbit	ND	128	ND	0
Monkey (Direct staining)	ND	≥4096	≥16,000	++++

[1] Sera kindly provided by Dr. *W. R. Clyde*, University of North Carolina School of Medicine.
[2] Reciprocal of serum dilution.

At times, perinuclear fluorescence was also seen. However, the nuclei of these infected cells were never involved and were always devoid of antigen. Besides the ciliated respiratory epithelial cells, no antigen was detectable in the alveolar cells of the lungs nor in the liver, spleen, or kidney. When a tissue was positive for antigen by FA staining, the infectivity titer was usually above 2.0 logs, ranging from 2.0—6.3 logs per 0.1 g

of tissues. In slightly over half of the hamster lung sections examined, there were some fluorescent leucocytes scattered in the interstitial areas. The fluorescence in these leucocytes was considered to be nonspecific as the fluorescence could not be inhibited by specific unlabeled Para 3 serum and many normal noninfected hamsters also showed these fluorescent leucocytes in their lung sections.

Starting on the 6th day after inoculation and thereafter up to the 14th day, all tissues examined became negative by FA staining except a trace of antigen was seen in the bronchial epithelium of the lungs from 2 hamsters sacrificed on the 6th day. However, the infectivity of these two lungs was below 1.0 log.

Pathogenesis in 3- and 7-day-old Hamsters

It is known that young infants are more susceptible to respiratory infections with more severe clinical illnesses. However, when 3-day or 7-day-old hamsters were used for infection, there did not seem to be much difference in the pathogenesis as com-

Table 5. Relationship of Hemagglutination-inhibition Antibody Titers in Anti-Parainfluenza 3 Sera on Direct and Indirect FA Staining of Para-3 Virus Infected Sections

FA conjugate designation No.	Serum prepared from:	Para-3 HI titer	Direct FA	Indirect FA at					
				10^1	20	40	80	160	320
32	Monkey, with oil adjuvant	≥ 4096[1]	++++	+	+	+	+	+	+
128	Rabbit, with Freund's adjuvant	5120	++++	++	+++	+++	+++	+++	++
45	Rabbit, without Freund's adjuvant	128	0	0	0	0	0	0	0
50	Rabbit, without Freund's adjuvant	640	±	0	±	0	0	0	0
56	Rabbit, without Freund's adjuvant	256	±	+	±	0	0	0	0
62	Rabbit, without Freund's adjuvant	256	±			ND			

[1] Reciprocal of serum dilutions.

Table 6. Effect of Cortisone Treatment on Parainfluenza

Days	Tissues									
	Nasal turbinate						Trachea			
	A[1]		B		C		A		B	
	FA[2]	TCD[3]	FA	TCD	FA	TCD	FA	TCD	FA	TCD
1	0	2.0	0	<1.0	0	<1.0	+	<1.0	0	<1.0
	0		0		0		0		0	
	0	1.0	0	<1.0	+	1.3	0	2.0	0	2.0
	0		0		0		0		0	
2	0	2.2	0	<1.0	0	2.8	0	1.7	±	<1.0
	0		0		0		0		±	
	0	1.5	+	2.7	+	1.6	0	3.3	+	4.3
	0		0		+		0		+	
3	0	3.7	0	3.7	0	3.3	±	3.5	++	3.5
	0		0		0		+++		+	
	+	2.5	+++	2.5	+	4.3	++	4.3	+++	4.8
	++		++		+++		++		+++	
4	+++	4.7	++	3.8	0	<1.0	+	3.0	++	4.3
	+++		0		+++		+++		+	
	+	2.3	+	2.6	0	3.3	++	4.5	+	3.6
	+		+		+++		+		+	
5	0	3.7	0	<1.0	0	1.0	++	3.0	0	1.7
	0		0		0		+		++	
	++	3.0	+	3.0	0	2.5	++	3.6	+	3.5
	+		+++		+		+		+++	
6	0	1.5	+++	2.0	+++	1.5	0	2.5	+++	4.3
	0		+		+++		0		+++	
7	0	1.0	0	1.0	ND	2.0	0	ND	+	0.7
	0		0		ND		0	ND	+	
	0	<1.0	+	1.5	++	2.6	0	<1.0	+	4.3
	0		+		+++		0		+	
8	0	<1.0	0	1.0	+++	2.5	0	<1.0	0	<1.0
	0		0		+		0		0	
9	0	1.0	0	1.0	0	1.7	0	<1.0	0	<1.0
	0		0		0		0		0	
	0	<1.0	0	<1.0	0	<1.0	0	<1.0	0	<1.0
			0		0				0	
11	0	<1.0	0	<1.0	0	<1.0	0	<1.0	0	<1.0
	0		0		0		0		0	
	ND	ND	0	<1.0	0	<1.0	ND	ND	0	<1.0
14	0	<1.0	0	<1.0	0	<1.0	0	<1.0	0	<1.0
	0		0		0		±		0	

[1] Group A = control; B = single dose of cortisone; C = multiple doses of cortisone.

[2] FA — fluorescent antibody staining; 0 = negative; + = minimum specific fluorescence; ++ = moderate specific fluorescence; +++ = maximum specific fluorescence.

Virus Type 3 Infection in Hamsters

Tissues								HI antibody[4]		
Trachea		Lung								
C		A		B		C				
FA	TCD	FA	TCD	FA	TCD	FA	TCD	A	B	C
0	<1.0	0	0.1	0	<1.0	0	1.0	ND	<4	16
0		0	1.0	±	1.5	0	1.0			
0	1.3	0	<1.0	0	<1.0	0	<1.0	<4	<4	<4
0		0	<1.0	0	<1.0	0	2.5			
0	1.5	0	1.5	0	1.8	0	1.3	<4	8	8
0		0	3.3	0	2.7	0	2.5			
++	3.4	0	<1.0	0	3.6	+	3.8	4	<4	<4
0		0	4.0	0	3.6	++	1.8			
0	1.0	0	<1.0	+++	4.3	0	2.2	8	4	<16
0		+++	4.5	+	1.5	0				
0	4.5	+++	4.4	+++	4.6	+++	2.7	<4	<4	<4
+++		+++	4.0	+++	4.0	+++	4.3			
0	<1.0	0	1.0	++	4.3	0	1.5	4	4	<4
0		++	4.7	0	3.2	+++	4.3			
0	5.0	++	4.0	++	3.7	+	3.3	<4	<4	<4
+++		+	3.5	+++	2.8	++	4.4			
0	3.7	++	2.7	0	1.5	0	2.0	4	<4	4
+++		++	3.5	++	3.5	+++	4.0			
	4.3	+++	3.4	+++	3.4	+++	3.4	<4	<4	<6
0		+++	3.5	+++	3.2	+++	4.2			
++	4.0	+	<1.0	++	3.5	+++	3.4	<4	<4	<4
+++		++	<1.0	+++	2.4	+++	4.4			
++	<1.0	0	<1.0	0	<1.0	+	1.0	16	4	<4
0		0	<1.0	±	<1.0	0				
+	3.0	0	<1.0	++	2.6	+++	<1.0	<4	<4	<4
+		0		+	<1.0	+++	2.7			
+++	3.3	0	<1.0	0	<1.0	+++	4.5	12	8	ND
+		0	1.4	0	1.5	++	2.5			
0	3.2	0	<1.0	0	<1.0	0	<1.0	256	256	4
++		0	<1.0	0	<1.0	0	3.5			
+	1.5	0	<1.0	0	<1.0	0	1.5	32	20	20
0				0	<1.0	0	<1.0			
0	<1.0	0	<1.0	0	<1.0	0	<1.0	64	64	16
+		0		0		0				
0	<1.0	ND	ND	0	<1.0	0	<1.0	ND	ND	ND
0	<1.0	0	<1.0	0	<1.0	0	<1.0	32	16	16
0		0		0	<1.0	0	<1.0			

[3] TCD — 50% tissue culture infectivity doses ($\log_{10}$) per 0.1 gram tissue.

[4] Hemagglutination inhibition antibody titer; reciprocal of serum dilutions. ND — no data.

Table 7. Viral Multiplication in Hamster Lungs and Development of Antibodies Following Intranasal Inoculation of Parainfluenza 3 Virus in Cortisone-treated and Untreated Hamsters

Days of infection	Infectivity titers and fluorescent antigen in lungs				HI antibody		Neutralization antibody	
	Control		Cortisone		Control	Cortisone	Control	Cortisone
4	2.3[1] (+)[2]	< 1.0 (+)	4.0 (+)	4.5 (++++)	< 4[3]	< 4	< 4	< 4
	4.0 (+)	3.3 (++++)	4.3 (++++)	4.6 (++++)	< 4	< 4	< 4	< 4
	3.3 (++++)	2.3 (++++)	5.0 (++++)	5.0 (++++)	< 4	< 4	< 4	< 4
8	< (0)[4]	< (+)	< (0)	0.6 (0)	128	32	10	5
	< (0)	< (0)	< (0)	< (+)	128	32	4	16
	< (0)	< (0)	< (+)	< (+)	128	128	25	128
12	< (0)	< (0)	< (0)	< (0)	128	64	32	< 2
	< (0)	< (0)	< (0)	< (0)	64	64	8	50
	< (0)	< (0)	< (0)	< (0)	256	256	5	16
16	< (0)	< (0)	< (0)	< (0)	512	512	80	40
	< (0)	< (0)	< (0)	< (0)	256	256	100	16
	< (0)	< (0)	< (0)	< (0)	256	64	32	16
20	< (0)	< (0)	< (0)		128	128	128	25
	< (0)	< (0)	ND		128	ND	100	ND
	< (0)	< (0)	ND		128	ND	40	ND

[1] Log_{10} TCD_{50} per 0.1 gram tissue.
[2] Degree of fluorescent staining, + minimum fluorescence; ++++ maximum fluorescence.
[3] Reciprocal of serum dilutions.
[4] < means infectivity titers less than 0.5 log.

pared to the use of 3- to 4-week-old animals in terms of the severity of infection and the amount of infectious virus recovered from lungs (Tables 2 and 3). Owing to the small size of these animals, the nasal turbinates and trachea were not collected for virus titrations. However, in microscopic examinations, there appeared to be more antigen detectable in the infected respiratory epithelium by FA staining (Fig. 6). Furthermore, examination of sections of larynx by FA staining in infected 3- to 4-week-old hamsters did not reveal any viral antigen, but the laryngeal epithelium of the infected 3- and 7-day-old hamsters did show the presence of viral antigen, however, the involvement in the larynx was not as severe as the nasal turbinates, the tracheas or the lungs.

Comparison of Direct and Indirect Methods of Staining

It is generally believed that if the direct method of fluorescent staining works in a given system, the indirect method should work equally well or even better. Human and animal sera known to contain Para 3 antibody as tested by CF, HI or neutralization tests were used for indirect staining. The results are shown in Table 4. To our surprise, none of these sera gave a positive FA staining on the infected lung sections which showed strong positive fluorescence when stained with our direct conjugate made from hyperimmune monkey serum.

With this unexpected finding, we decided to conduct a systematic investigation on the comparison of direct and indirect FA staining on Para 3 infected tissues. Several batches of rabbit sera prepared with or without Freund's adjuvant and a monkey hyperimmune serum were used. A portion of the sera was precipitated and labeled with fluorescein isothiocyanate for direct staining. Samples of unlabeled sera were titrated for HI antibody titer and indirect fluorescent staining on Para 3 infected hamster lungs. The results are summarized in Table 5.

As seen in Table 5, only two sera, one from monkey, the other from rabbit, prepared with adjuvants and having an HI titer of $\geq$4096 and 5120 respectively gave a satisfactory labeling to be used as direct conjugate for Para 3 staining. The rest of the sera prepared without adjuvants having HI titers between 128—640 did not make satisfactory conjugates for direct staining. Similarly, only the first two sera prepared with adjuvants gave positive indirect staining, while the other sera did not. In performing the indirect FA staining, we used various dilutions of unlabeled sera to rule out any possible "zone effect". However, the monkey serum gave a very poor staining and we attributed this difficulty partly as being due to poor preservation from repeated freezing and thawing of the same serum for various other tests. However, even in the rabbit serum which had not been frozen and thawn repeatedly, the indirect staining quality from dilutions 1/10 to 1/160 was never as clear and sharp

as its corresponding direct conjugate. At low dilutions, 1/10 and 1/20, the background nonspecific fluorescence was quite noticeable.

From these data, we concluded that the indirect FA technic in detecting Para 3 viral antigen in infected tissues is inferior to the direct FA technic. Furthermore, hyperimmune sera with high HI titers greater than 1/640 are necessary in preparing satisfactory conjugates for direct staining.

Effect of Cortisone on the Course of Infection

Three groups of hamsters, 30 in each group, were used for these experiments. All hamsters were of about the same size, weighing between 50—60 g.

Group A — controls, no cortisone was given.

Group B — one single injection of 2.5 mg cortisone acetate (Merck, Sharp and Dohme) was given intramuscularly (i.m.) 24 hours before intranasal inoculation of virus.

Group C — in addition to 2.5 mg of cortisone as in Group B, each hamster in this group also received 1.0 mg cortisone acetate i.m. every other day. For instance, the hamsters sacrificed on the 9th day after virus inoculation had received a total of 6.5 mg of cortisone.

Hamsters in all three groups received the same amount of virus intranasally as in previous experiments. Two hamsters from each group were sacrificed daily and the results from two separate experiments are summarized in Table 6.

There were no overt signs of infection in either controls or cortisone-treated animals. The pattern of viral antigen distribution in the respiratory epithelium as demonstrated by FA staining also did not differ in these three groups of animals. However, in Group C animals which received multiple doses of cortisone acetate, there seemed to be fewer leukocytes present in the lung sections as compared to the control animals. The amounts of infectious virus in infected tissues of cortisone treated and untreated animals were the same. However, in cortisone treated animals, the persistence of virus as measured by infectivity titrations and by FA staining was longer than in the untreated hamsters.

As shown in Table 6 in Group A control animals, virus was present in the respiratory tissues up to the 5th day of infection. Although the bronchial epithelium still showed the presence of some viral antigen on the 6th day, the infectivity titrations of these lungs were below 1 log. In Group B, where hamsters received only a single injection of 2.5 mg cortisone, virus was detectable up to the 7th day of infection. In Group C, where hamsters received multiple doses of cortisone, unequivocal persistence of virus was demonstrated up to the 9th day of infection as shown by 1.5 to 3.5 logs of virus recovered from the tissues in more than half

of the inoculated animals and the presence of viral antigen by FA staining in the trachea in 2 out of 4 hamsters. On the 11th day of infection, although all virus titrations were negative (below 1 log), some viral antigen was still visible in the trachea in 1 out of 3 animals.

Development of Antibodies Following Infection

The development of hemagglutination-inhibition (HI) antibody was measured in cortisone-treated and untreated animals. Pooled sera, from the two daily sacrificed hamsters in each of the three groups were titrated for HI antibody with sera inactivated at 56° C for 30 minutes and pretreated with KIO_4. There was no significant difference in HI antibody titers among all three groups, although perhaps the group with multiple cortisone injections might show a trend of delayed rise within the observation period of 14 days (Table 6).

To ascertain more precisely whether cortisone treatment had any effect on antibody production following Para 3 infection, an experiment was performed as follows:

Two groups of hamsters, similarly treated as in Group A (control, no cortisone) and Group C (multiple doses of cortisone) in the previous experiment were used. Beginning on the 4th day following intranasal inoculation and every 4th day thereafter, 6 hamsters from each group were sacrificed. The lungs from each hamster were titrated for virus and frozen sections were cut for FA staining. Sera from two hamsters were pooled and tested for HI and neutralization antibody titers. The results are summarized in Table 7.

It can be seen that in this experiment, practically all hamsters developed a positive infection following inoculation of Para 3 virus. At 4 days, all lung titrations except one in the control group showed viral multiplication and FA staining showed presence of antigen in the epithelial cells. The virus titers in the cortisone-treated animals seemed to be somewhat higher than in the controls. In both groups of inoculated hamsters, the HI and neutralizing antibodies began to appear on the 8th day of infection and thereafter. The titers did not differ significantly between cortisone-treated or not treated animals.

Experiments were also conducted to measure interferon production. Lungs were harvested daily from Para 3 infected hamsters with and without cortisone treatment from the first day to the 14th day of infection. A 10% suspension of the lungs was made in buffered saline. Interferon preparation was made according to the method of *Gifford* et al. (12) by first centrifuging the suspension at 18,000 r.p.m. for 1 hour, followed by dialysis at pH 2 buffer for 24 hours. These preparations were tested for interferon activity in hamster kidney cells either by suppression of cytopathogenic effect (CPE) or by reduction of plaque counts using Sind-

bis virus as indicator. No interferon activity was demonstrable in the preparations when tested by suppression of CPE in hamster kidney cells infected by Sindbis virus. However, by the plaque reduction method, taking a 50% or greater reduction as significant, interferon production appeared as early as one day after infection in both groups of animals and continued to be present up to the 9th day after infection. There was no significant difference of interferon content in the lungs of these two groups of hamsters.

Discussion

Under the existing experimental conditions, intranasal inoculation of type 3 parainfluenza (Para 3) virus in hamsters did not produce obvious signs of illness nor appreciable pathological changes in the respiratory tract when examined by conventional histological methods. However, infectivity titrations of tissues showed unequivocal viral multiplication and immunofluorescent staining of tissue sections showed an extensive antigenic involvement of infection in the respiratory epithelium. The infection was self-limited with elimination of infectious virus and disappearance of viral antigen at about 5 to 6 days after infection followed by a prompt antibody response detectable both by hemagglutination-inhibition and neutralization in the serum of infected animals. In these respects the experimental infection in animals simulates the natural disease in human beings. Other investigators have also reported that parainfluenza viruses usually multiply in the respiratory tracts without producing overt illness in guinea pigs and hamsters inoculated with such viruses intranasally (4, 13—15).

Our observations, showing the localization of parainfluenza 3 viral antigen by immunofluorescent staining only detectable in the cytoplasme and not in the nuclei of infected cells, are in agreement with observations reported by *Maassab* and *Loh* (16), and at variance with those of *Cohen* et al. (17). Perhaps this might be due to differences of virus strains and tissue culture cell types used in different experiments. It is interesting to note that among the myxoviruses, some, such as influenza (18) and measles (19) show viral antigens in the nuclei of infected cells sometime during their multiplication cycles, while others, such as parainfluenza, mumps and Newcastle viruses do not have viral antigen demonstrable intranuclearly (20).

With large multiple doses of corticosteroids administration, there was a prolongation of infection as evidenced by persistence of infectious virus and viral antigen in tissues 9—11 days after inoculation. Although there was no increase of the severity of infection and of the amount of viral growth and antigenic distribution in infected tissues, the prolongation of viral persistence in respiratory tissues was significant.

It is not clear by which mechanism cortisone administration prolongs the viral persistence in infected animals. Neither the hemagglutination-inhibition nor the neutralizing antibody was inhibited by cortisone treatment. Using methods available to us for measuring interferon activities in infected hamster lungs, we could not demonstrate a suppression of interferon production in the lungs of cortisone-treated animals. Pharmacologically, cortisone has an anti-inflammatory effect, and in those cortisone-treated hamsters there seemed to be fewer fluorescent macrophages present in the lung sections; whether the suppression of phagocytosis by cortisone has altered the host resistance is speculative. However, *in vitro* phagocytosis of influenza virus by rabbit leukocytes has been reported (21), we believe further investigations in regard to phagocytosis of parainfluenza virus *in vivo* is warranted.

The data here suggest quite clearly that there exists a direct relationship between the serum antibody titer against parainfluenza 3 virus and its effectiveness in immunofluorescent staining. Serums with antibody titer sufficient for conventional serological reactions may not be potent enough for preparation of fluorescent conjugates. Furthermore, the direct fluorescent antibody staining technic is superior to the indirect method in detecting parainfluenza 3 viral antigens in infected tissue sections. Similar experiences have been encountered in other systems (19). Therefore, an investigator should be careful in choosing his immunofluorescent tool for his work and should not rely on the belief that the indirect fluorescent staining nethod works equally as well or better than the direct method.

Summary

After intranasal inoculation of parainfluenza type 3 virus in hamsters, viral multiplication was detectable by infectivity titrations and immunofluorescent (FA) staining to have occurred in respiratory tissues as early as 24 hours following inoculation. Virus persisted in the respiratory epithelium up to the 6th day of infection. By FA staining, localization of viral antigen was seen in the cytoplasm of infected ciliated epithelial cells in the form of brilliant fluorescent granules of varying size.

Administration of corticosteroids to hamsters prolonged the parainfluenza infection to 9 to 11 days after virus inoculation. However, the amount of infectious virus and antigenic distribution showed no significant difference between treated and untreated animals.

The direct FA staining was found to be superior to the indirect method in demonstrating the antigenic localization. The reaction was specific and no cross reaction was encountered with other myxoviruses tested. Hyperimmune animal serum with high antibody titers against parainfluenza 3 virus was required to prepare satisfactory fluorescent conjugates.

References

1. *Dick, E. C., W. J. Mogabgab,* and *B. Holmes:* Characteristics of parainfluenza I (HA2) virus. I. Incidence of infection and clinical features in adults. Amer. J. Hyg. **73,** 263 (1961).
2. *Parrott, R. H., A. J. Vargosko, H. W. Kim, J. A. Bell,* and *R. M. Chanock:* Acute respiratory diseases of viral etiology. III. Myxovirus: Parainfluenza. Amer. J. publ. Hlth **52,** 907 (1962).
3. *Chanock, R. M., R. H. Parrott, K. M. Johnson, A. Z. Kapikian,* and *J. A. Bell:* Myxoviruses: Parainfluenza. Ann. Rev. resp. Dis. **88,** 152 (1963).
4. *Craighead, J. E., M. K. Cook,* and *R. M. Chanock:* Infection of hamsters with parainfluenza 3 virus. Proc. Soc. exp. Biol. (N.Y.) **104,** 301 (1960).
5. *Wenner, H. A., C. A. Miller, P. Kamitsuka,* and *J. C. Wilson:* Preparation and standardization of antiserums prepared with the three known types of poliomyelitis viruses. Amer. J. Hyg. **59,** 221 (1954).
6. Committee on Standard Serological Procedures in Influenza Studies. An agglutination-inhibition test proposed as a standard of reference in influenza diagnostic studies. J. Immunol. **65,** 347 (1950).
7. *Riggs, J. L., R. J. Seiwald, J. H. Burckhalter, C. M. Downs,* and *T. G. Metcalf:* Isothiocyanate compounds as fluorescent labeling agents for immune serum. Amer. J. Path. **34,** 1081 (1958).
8. *Weller, T. H.,* and *A. H. Coons:* Fluorescent antibody studies with agents of varicella and herpes zoster propagated *in vitro*. Proc. Soc. exp. Biol. (N.Y.) **86,** 789 (1954).
9. *Coons, A. H., E. H. Leduc,* and *M. H. Kaplan:* Localization of antigen in tissue cells. VI. The fate of injected foreign protein in the mouse. J. exp. Med. **93,** 173 (1951).
10. *Chanock, R. M.,* and *K. M. Johnson:* Parainfluenza viruses, in diagnostic procedures of viral and Rickettsial infection. *E. H. Lennette* and *N. J. Schmidt,* eds. Amer. publ. Hlth Ass. Inc. 3rd edition, p. 475 (1964).
11. *Reed, L. J.,* and *H. Muench:* A simple method of estimating 50% endpoints. Amer. J. Hyg. **27,** 493 (1938).
12. *Gifford, G. E., S. T. Toy,* and *J. Lindenmann:* Studies on Vaccinia virus plaque formation and its inhibition by interferon. Virology **19,** 294 (1963).
13. *Cook, M. K., B. E. Andrews, H. H. Fox, H. C. Turner, W. D. James,* and *R. M. Chanock:* Antigenic relationships among the "newer" myxoviruses (parainfluenza). Amer. J. Hyg. **69,** 250 (1959).
14. *Johnson, K. M., R. M. Chanock, M. K. Cook,* and *R. J. Huebner:* Studies of a new human hemadsorption virus. 1. Isolation, properties and characterization. Amer. J. Hyg. **71,** 81 (1960).
15. *Cook, K. M.,* and *R. M. Chanock: In vivo* antigenic studies of parainfluenza viruses. Amer. J. Hyg. **77,** 150 (1963).
16. *Maassab, H. F.,* and *P. C. Loh:* Fluorescent antibody studies in tissue culture of parainfluenza 3 infection. Proc. Soc. exp. Biol. (N.Y.) **109,** 897 (1962).
17. *Cohen, S. M., S. Bullivant,* and *G. A. Edwards:* A morphologic study of FL cells infected with parainfluenza 3 virus. Arch. ges. Virusforsch. **21,** 493 (1961).
18. *Liu, C.:* Studies on influenza infection in ferrets by means of fluorescein-labelled antibody. II. The role of "soluble antigen" in nuclear fluorescence and cross-reactions. J. exp. Med. **101,** 677 (1955).

19. *Llanes-Rodas, R.*, and *C. Liu:* A study of measles virus infection in tissue culture cells with particular reference to the development of intranuclear inclusion bodies. J. Immunol. **95,** 840 (1965).
20. *Traver, M. I., R. L. Northrop*, and *D. L. Walker:* Site of intracellular antigen production by myxoviruses. Proc. Soc. exp. Biol. (N.Y.) **104,** 268 (1960).
21. *Hanson, R. J., J. E. Kempf*, and *A. V. Boand:* Phagocytosis of influenza virus. II. Its occurrence in normal and immune mice. J. Immunol. **79,** 422 (1957).

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