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THE INTESTINE OF FERRET — A POSSIBLE SITE OF INFLUENZA VIRUS REPLICATION

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Summary. — Anal virus shedding and stimulation of the immune response were observed in ferrets after oral and rectal administration of influenza A/Hongkong/1/68 (H3N2) virus. Despite of the low pH in the stomach, the virus had reached the intestines after oral administration and was found in high concentration in faeces and in mucosal cells lining the colon. Using immunofluorescent staining, the virus antigen was detected in cytoplasm of columnar epithelial cells. Virus replication also occurred in the respiratory tract, probably as result of inhalation of anally shedded virus. However, the virus replication in the lungs did not seem essential for virus isolation from the intestines. Taking into account the very short food passage time in ferrets the results could indicate the replication of influenza A/Hongkong/1/68 virus in the digestive tract of ferrets.

Key words: influenza virus; ferret intestine; serum; antibody virus replication

Introduction

The intestine of feral and domestic ducks has been recently shown to represent the major replication site of avian influenza viruses by experimental studies and under natural conditions (Slenoms and Easterday, 1978; Webster *et al.*, 1978; Hinshaw *et al.*, 1979; Kida *et al.*, 1980). The replication of avian and human influenza viruses in organ cultures of chick colon was also reported (Ohta *et al.*, 1981). Although enteritis has been observed in human influenza (Douglas, 1975), there are no reports of virus isolations from alimentary tract tissues lower than the oesophagus in man, ferrets or other mammals (Toms *et al.*, 1974; Rosztoczy *et al.*, 1975). It is well-known that mammalian influenza A viruses are more acid labile than avian influenza A viruses (Chagnon *et al.*, 1965; Glathe *et al.*, 1982) and therefore, gastric acid seems to be a barrier preventing infection of the lower intestinal tract. On the other hand, oral immunization with live influenza virus has been shown effective in stimulation of immune response in man as well as in laboratory mammals (Aleksandrova *et al.*, 1970; Boudreault and Pavilanis, 1972; Sidorova and

Alekseeva, 1973; Boudreault *et al.*, 1976; Alekseeva *et al.*, 1979; Koval *et al.*, 1979; Bergmann and Waldman, 1982). In our study we investigated the site of virus replication and stimulation of immunological response in ferrets after oral and rectal administrations of influenza virus A/Hongkong/1/68.

Materials and Methods

Animals. Ferrets, male and female (1.0—1.5 kg) were bought from a commercial source (Fa. Gottschalk, Karl-Marx-Stadt, G.D.R.). They were housed in single cages in an animal house without barrier-system.

Virus. The prototype influenza strain A/Hongkong/1/68 (H3N2) was obtained from the Influenza Centre of G.D.R. and was passed once in 11-day-old embryonated eggs. The allantoic fluids were harvested after 48 hr incubation at 35 °C, clarified in a bench centrifuge and stored at —70 °C in small aliquots.

Virus inoculation. Oral inoculation was done by intragastric tubing with a plastic tube. Each animal received 1.0 ml virus suspension (10^3 or 10^4 EID_{50}). After virus inoculation, the plastic tube was washed *in situ* with 3.0 ml phosphate buffered saline (PBS). For rectal inoculation 1.0 ml virus suspension was deposited directly into the rectum. Before virus inoculation the ferrets were anaesthesized by intraperitoneal injection of sodium hexabarbital, 25 mg/kg body weight.

Sample collection. Anal samples and nasal washings were collected as described previously (Webster *et al.*, 1978) and inoculated into the allantoic cavities of 11-day-old embryonated eggs. Five eggs were used per sample. Haemagglutinating activities of allantoic fluids were tested after 48 hr incubation at 36 °C. Positive samples were frozen at —70 °C for further investigations.

The ferrets were killed for virus isolation from organs and for histological examination by bleeding through the carotis in anaesthesia at various times after virus inoculation. Blood, lungs, turbinates, spleen, duodenum, jejunum and colon were removed aseptically.

The contents of the intestines (duodenum, jejunum, colon) were collected, diluted with buffered glycerol-saline with antibiotics to yield suspensions of 10% (v/v) and centrifuged (10 000 g, 60 min). Virus was recovered as described above. Parts of the intestines were frozen immediately with liquid nitrogen. The segments of intestines were then cut open and washed extensively with PBS. Musocal cells were removed by scraping with a blade. The harvested cells were suspended with buffered glycerol saline to yield suspensions of 10% (v/v) and disrupted by freezing and thawing. After low speed centrifugation in a bench centrifuge the supernatans were harvested and virus assay was performed.

Extracts of lungs, turbinates and spleen were prepared by grinding in a mortar in the presence of sterile sand. 10% (w/v) suspensions were prepared with buffered glycerol saline. Virus recovery from blood was performed directly. The viruses recovered from each sample were identified by haemagglutination-inhibition test (HIT) with post-infection ferret antiserum to A/Honkong/1/68 virus. Virus titres were calculated by the method of Reed and Muench (1938) and expressed as EID_{50} per gram of tissue or 1.0 ml of fluid.

Virus challenge. Ferrets were infected intranasally with 10^7 EID_{50} of A/Hongkong/1/68 virus three weeks after the oral virus administration.

Haemagglutination inhibition (HI) antibody titration. Blood samples were drawn from the sublingual vein prior to and 3 weeks after infection. The sera were treated with KIO_4 and assayed using a standard micromethod with 4 haemagglutinating units of antigen and 0.5% chicken erythrocytes in plastic microtitre trays.

Immunofluorescent staining. Cryostat sections (Cryocut II, Reichert-Jung, Wien) 5 μm thick from the ferret intestine were dried at room temperature and fixed with acetone for 15 min. Each tissue section was overlaid with the immunoglobulin fraction of rabbit antiserum to A/HongKong/1/68 in a moist chamber for 30 min and washed three times in PBS. The sections were stained with the fluorescein-isothiocyanate labelled immunoglobulin fraction of goat antiserum to rabbit immunoglobulin (State Institute for Media and Immunpreparations, Berlin, G.D.R.) in moist chamber and washed again in PBS. Finally the sections were counterstained with a solution of 0.001% Evans blue, washed again in PBS and mounted into buffered glycerol (pH 9.0).

Table 1. Immune stimulation and anal virus shedding after oral application of influenza A/ /Hongkong/1/68 virus in ferrets

No.	Dose EID_{50}	Anal virus shedding[1]) on day 1	2	3	4	Antibody titre[2]) on day 1	21	After challenge (day 21) Nasal virus shedding[3]) on day 22	23	Virus content[4]) Turbinates	Lungs
7	10^8	4/7	4/7	3/7	3/7	3.7 ± 0.8	6.9 ± 1.2	1.7 ± 0.5	<2.0	2.6 ± 1.0	< 2.0
4	10^4	0/4	1/4	2/4	1/4	3.3 ± 0.5	6.5 ± 0.6	2.0 ± 1.0	< 2.0	2.9 ± 1.0	< 2.0
4	Contact	0/4	0/4	0/4	0/4	2.5 ± 1.0	6.0 ± 1.1	< 2.0	< 2.0	2.3 ± 1.3	< 2.0
3	Control	n.t.	n.t.	n.t.	n.t.	2.3 ± 0.6	2.7 ± 1.2	3.4 ± 1.0	3.7 ± 0.8	5.8 ± 0.5	2.8 ± 1.4

[1]) Number of positive samples/number of ferrets infected.
[2]) Mean antibody titre ($\log_2$) $\pm$ standard error
[3]) log EID_{50}/ml
[4]) log EID_{50}/g
N = number of animals.

The sections were examined in microscope HBO 202, Fluoval I (VEB Carl Zeiss, Jena). The following controls were used: 1. Sections of noninfected ferrets, 2. Sections of infected ferrets stained with heterologous antiserum (rabbit antiserum to parotitis virus), 3. Sections of infected ferrets stained with the fluorescein isothiocyanate-labelled anti-rabbit immunoglobulin only.

Results

In the first experiment (Table 1) 7 ferrets were inoculated orally with 10^8 EID_{50} of A/Hongkong/1/68 virus and 4 ferrets received 10^4 EID_{50}. Four contact animals were kept side by side in separate cages, 3 animals served as controls and were housed in a separate room. Three weeks later all animals were infected intranasally with 10^7 EID_{50} of the same virus. After oral virus inoculation no clinical symptoms of respiratory illness were observed but in all ferrets diarrhoea was noted lasting for at least three days. The virus was recovered from the faeces of 4 out of 7 ferrets inoculated with 10^8 EID_{50} of virus from the first day post-inoculation (p.i.).

In the group inoculated with 10^4 EID_{50} of the virus, anal shedding was detected in 1 out of 4 ferrets from the second day p.i. Anal virus shedding

Table 2. Site of A/Hongkong/1/68 (H3N2) virus replication after oral inoculation of ferrets

Days p.i.	Nasal washing	Anal swabs	Blood	Lungs	Spleen	Duodenum Content	Duodenum Cells	Jejunum Content	Jejunum Cells	Colon Content	Colon Cells
1	5/8*	0/8	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
2	5/6	0/6	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
3	4/4	4/4	1/2	2/2	1/2	1/2	1/2	1/2	1/2	1/2	2/2
4	2/2	2/2	1/2	2/2	0/2	0/2	0/2	0/2	1/2	2/2	2/2

* No. of positive samples out of ferrets infected.

Table 3. Virus titres after oral application of influenza A/Hongkong/1/68 virus in ferrets[1])

	Ferret No[2])	Anal swabs	Lungs	Blood	Duodenum		Jejunum		Colon	
					Cells	Content	Cells	Content	Cells	Content
	1	3.1	2.0	< 2.0	< 2.0	< 2.0	2.5	< 2.0	2.3	2.7
1	2	5.8	2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	4.5	4.5

-) $\log_{10}$ EID_{50}/g tissue or ml fluid.
[2]) Material collected on day 4 after virus inoculation.

was observed also in 2 ferrets inoculated with 10^8 EID_{50} of virus until day 6 (data not shown). The rise in mean antibody titre was more than 4 fold in both groups orally inoculated as well as in the contact group. The quantities of virus recovered after challenge from lungs and turbinates in the orally inoculated group and controls indicate a strong local and systemic immunity after oral virus administration. In this respect the reaction of the contact animals was quite the same as seen in the orally inoculated ones. Thus the anal shedding of live influenza virus was confirmed not only by virus recovery from the faeces but also by spreading to the animals kept in close contact.

In the second experiment (Table 2), 8 ferrets were inoculated orally with 10^8 EID_{50} of A/Hongkong/1/68 virus. On the following 4 days nasal washings and faeces were collected. Two ferrets were sacrificed daily and organs were removed for virus recovery and immunofluorescent staining. In this experiment anal virus shedding was detected on day 3 and 4 p.i. From nasal washings the virus was isolated in 5 out of 8 ferrets on the first day and in all 4 animals remaining on day 3. The virus was recovered from the intestine of the ferrets sacrificed on day 3 and 4. On these days virus was present in all colon cell supensions. Moreover, in ferrets killed on day 3 and 4 the virus was also present in the jejunum. Virus recovery from the duodenum was successful only in one ferret. Virus was also isolated from the lungs on day

Table 4. Site of A/Kongkong/1/68 (H3N2) virus replication in ferrets after rectal inoculation

Days p.i.	Nasal washing	Anal swabs	Blood	Lungs	Spleen	Duodenum[1])	Jejunum[1])	Colon[1])
1	3/8*	6/8	0/2	0/2	0/2	0/2	1/2	1/2
2	4/6	3/6	0/2	0/2	0/2	0/2	1/2	2/2
3	3/4	3/4	0/2	0/2	0/2	0/2	0/2	2/2
4	n.t.	2/2	0/2	0/2	0/2	1/2	0/2	2/2

Mean antibody titre ($\log_2 \pm$ standard error) n = 4): day 1 = 4.0;
day 21 = 6.5 ± 0.9

[1]) Content of intestinal segments
n.t. = Not tested
* No. of samples out of ferrets infected.

Table 5. Results of virus isolation and immunofluorescent staining after oral or rectal inoculation of A/Hongkong/1/68 virus in ferrets

Days p.i.	Route of inoculation: Oral						Rectal					
	Isolation			Imunofluorescence			Isolation			Immunofluorescence		
	D[1])	J[2])	C[3])	D	J	C	D	J	C	D	J	C
1	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	+	+	−	+	+
2	−	−	−	−	−	−	−	+	+	−	+	+
	−	−	−	−	−	−	−	−	+	−	−	+
3	−	−	+	−	−	−	−	−	+	−	−	+
	+	+	+	±	+	+	−	−	+	−	−	−
4	−	+	+	−	+	−	−	−	+	−	−	−
	−	−	+	−	−	+	−	−	+	+	−	−

[1]) D = duodenum [2]) = jejunum [3]) C = colon

3 and 4. On these days virus was also present in the blood of two and in the spleen of one animal.

The virus content of the organs and the faeces of two ferrets killed on day 4 is shown in Table 3. The virus titres were high in the faeces and in the lower intestines.

These experiments showed that the A/Hongkong/1/68 virus can pass through the intestinal tract despite of the low pH values in the stomach. However, it remained unclear whether virus replication in the respiratory tract was necessary to establish infection of the intestine. To answer this question 12 ferrets were inoculated directly into the rectum (Table 4). The results revealed that rectal inoculation had bypassed infection of the lower respiratory tract. Virus was not isolated from lung, spleen and blood. On the first day after inoculation virus was recovered from the faeces of 6 out of 8 ferrets and also from one colon and jejunum. Virus was detected in all colon specimens collected on day 2, 3 and 4 and in one duodenum (day 4). No isolation attempts were made from intestinal tract suspensions. Antibody determinations in 4 animals exhibited a 4-fold rise in HI antibodies. The intestines of the ferrets included in the second and third experiment were also examined by immunofluorescent staining.

Only the organs from which the virus was isolated were positive in both studies (Table 5). In some cases detection of virus antigen by immunofluorescent staining seems to be less sensitive than virus isolation. Fig. 1 shows bright

specific fluorescence in the columnar epithelial cells which formed crypts in the colon of ferret no. 6 of the second study. Bright specific fluorescence in the epithelial cells of the lower part of the jejunum of ferret no. 5 in the same experiment is demonstrated in Fig. 2. Both figures reveal intensive cytoplasmatic fluorescence. In general, fewer positive cells were observed in sections of the jejunum than in the colon. Specific but very weak fluorescence was observed also in the duodenum of two ferrets.

Discussion

The results of our studies could indicate replication of influenza A/Hongkong/1/68 virus in the intestinal tract of ferrets. Because most positive results were obtained from the content and the mucosal cells of the colon this part of intestine may be the possible site of virus replication. The replication of avian influenza viruses in the lower intestinal tract of feral and domestic ducks was demonstrated earlier (Slenoms and Easterday, 1978; Webster *et al.*, 1978). Until now there is no evidence that human or other mammalian influenza viruses will replicate in the interstinal tract of their natural hosts. Attempts for demonstration of influenza virus replication in organ cultures of intestinal tract tissue of ferrets failed (Toms *et al.*, 1974). But these authors used the A/Moscow/1019/65 (H2N2) strain, therefore it is not possible to compare the results of this survey with our study. Other investigators (Boudreault and Pavilanis, 1972; Sidorova and Alekseeva, 1973) made no attempts to isolate virus from faeces or intestinal tract material. Aleksandrova *et al.* (1970) found a slight enlargement of the regional lymph nodes as a result of virus multiplication in Waldayer's pharyngeal lymphatic ring, since virus was isolated regularly from the nasopharynx after oral vaccination of volunteers. We also found the inoculated virus in the respiratory tract. These findings may be the result of nasal uptake of anal shedded virus and the aspiration of small amounts of virus after oral application.

Influenza virus was isolated from faeces and intestinal contents not only after oral virus administration but also after virus inoculation into the rectum. Therefore, it is not likely that anal virus shedding is the result of intensive virus multiplication outside the intestinal tract. Virus antigen was also detected in the cytoplasm of mucosal cells by indirect immunofluorescent staining. The food passage time in ferrets was found to be rather short (180 min) (Bleavins and Aulerich, personal communication). Thus the rapid passage of virus mixed with food through the digestive tract must permit at least a proportion of the virus from being exposed to the low pH and bile salts. The signs of enteral disease in the orally infected ferrets support the concept of virus multiplication in the intestinal tract. The question whether this phenomenon is typical only for the A/Hongkong/1/68 strain or other H3N2 strains remains open so far.

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Refereces

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Explanation of Micrographs (Plate XLVI):

Fig. 1: Virus antigen in musocal cells lining the crypts in colon (x 360).

Fig. 2. Virus antigen in mucosal cells lining the crypts in jejunum (x 360)