

## Dissemination of Influenza Virus Between Anatomically Isolated Sites in Ferrets

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Influenza virus was shown to disseminate from the respiratory tract of nasally infected ferrets to a surgically formed, subcutaneous tracheal pouch. Conversely, ferrets infected via the pouch shed virus from the upper respiratory tract. These events occurred within the first 24 h after infection. Passively administered ferret antibody to virus did not prevent the dissemination.

The primary purpose of this research was to try to establish a model for the study of local or secretory host defense mechanisms against influenza infection. The development of the tracheal pouch technique (25), wherein a segment of trachea is isolated and implanted subcutaneously in the neck region of the same animal, presents two anatomically separate sites for infection (Fig. 1). This technique permits the evaluation of different local host defenses in the same animal and thereby may possibly better define local or secretory immunity. The ferret was chosen as the experimental animal because influenza in ferrets closely resembles the disease in man (14). Since influenza virus replicates readily in respiratory epithelium (2, 4-6) but is found infrequently in blood (2, 9, 10, 13, 15, 20, 23), it was postulated that infection of one site might be restricted to that site or, alternatively, if not restricted, that passive antibody would inhibit the dissemination of the virus from one site to the other. The results presented in this paper demonstrate that virus can be isolated from both sites irrespective of which site was originally infected, and that passive serum antibody does not appear to inhibit this dissemination.

### MATERIALS AND METHODS

**Virus.** The virus used in all phases of the experiment was an A<sub>0</sub> strain of influenza obtained from Robert Waldman, University of Florida, College of Medicine, Gainesville. The virus was propagated in the allantoic cavity of 10-day-old embryonated eggs, harvested, and stored in glass ampoules at -70 C. The virus pool had a 50% egg infective dose at  $3.3 \times 10^8$ /ml and a hemagglutination (HA) titer of 1:256 for chicken erythrocytes.

**Ferrets.** Adult male ferrets, not previously exposed to influenza virus, were obtained from Marshal Research Animals, Inc., North Rose, N.Y. The animals were cared for in accordance with public laws 89-544

and 91-579, as amended. All animals were bled on arrival and were shown to have no detectable influenza antibody levels (hemagglutination inhibition [HI] titer <1:8). The ferrets were housed individually in separated cages within a ventilated hood at all times. Unpublished work has previously established that ferrets housed in this manner did not become infected when animals in adjacent cages were infected with influenza virus.

**Passive antiserum to influenza.** Six ferrets, previously infected with influenza virus, were inoculated subcutaneously with 0.2 ml of the undiluted virus pool. All animals were bled 16 to 19 days later to obtain 45 ml of whole blood. The sera were found to have an HI titer of 1:512.

**Titrations.** Microtiter loops, pipettes, and v-plates (Cooke Engineering Co., Alexandria, Va.) were used for all titrations.

**(i) HI titers.** New microtiter plates were used to avoid false readings from improperly cleaned plates (17). A dextrose-gelatin-Veronal (DGV) buffer, pH 7.3, was used as a diluent (1, 15). Kaolin (acid-washed American Standard, Fisher Scientific Co., Fair Lawn, N.J.) was prepared as a 25% suspension in a borate saline solution and stored at 4 C (1). Prior to use, the kaolin was sedimented by centrifugation and resuspended in DGV at a pH of 7.4 (8).

Adult chicken erythrocytes were stored for up to 2 weeks at 4 C in Alsever solution prior to use and suspended at 0.16% in DGV for use in HI and HA tests (16). The technique for absorbing and titering sera was that described by Sever et al. (18).

**(ii) HA titers.** To 0.025-ml serial twofold dilutions of virus in DGV was added 0.025 ml of a 0.16% suspension of adult chicken erythrocytes. Trays were sealed and placed at 4 C overnight, and results were recorded the following day (18, 24).

**Tracheal pouch.** A slight modification of the technique described for dogs by Wardell et al. (25) was used to implant physiologically functional, anatomically isolated tracheal pouches beneath the integument in ferrets. No stoma into the pouch lumen was made, as Wardell described, and 4-0 stainless-steel wire was employed to fix the pouch in a permanent location and to facilitate aspiration. Of 10

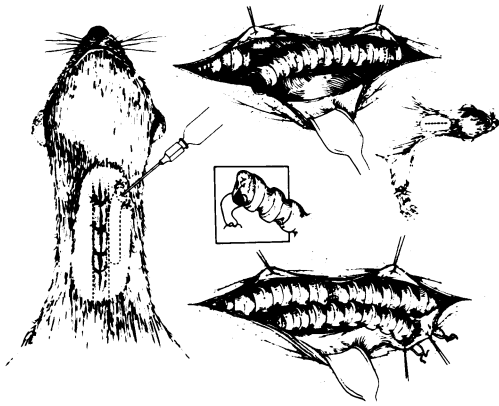


FIG. 1. Schematic representation of tracheal pouch operation and aspiration. Upper left: mid-line incision exposing trachea and transection of trachea to isolate eight tracheal rings. Center: closure of caudal end of pouch. Upper right: anastomosis of trachea and suturing of cephalad end of displaced tracheal pouch to under surface of skin. Bottom: percutaneous aspiration of pouch.

ferrets originally receiving tracheal pouches. 2 died of operative complications. One death was attributed to an overdosage of pentobarbital anesthesia, and subsequently Ketamine-hydrochloride (approximately 150 mg/kg) intramuscularly administered, was found to be a better anesthetic agent. The other ferret developed a mucus plug at the site of anastomosis and was found asphyxiated 10 days after surgery.

**Collection of fluid samples.** All blood samples were taken by cardiac puncture by using a disposable 22-gauge needle. Samples of nasal washings were obtained by injecting 0.5 to 0.8 ml of physiological saline (0.85% NaCl) into an animal's nostrils and then allowing it to expel the fluid mixed with mucus into a sterile container. By using Ketamine anesthesia (approximately 75 mg/kg), the tracheal pouches were aseptically aspirated daily beginning 14 days post-surgery and by first washing the area of skin surrounding the puncture site with soap and then placing a 19-gauge needle into the pouch lumen and withdrawing the contents into a syringe. In this way, 0.05 to 0.3 ml of tracheal secretions was usually obtained from each animal. About once in five attempts the above procedure failed to yield fluid. Then a second needle was inserted into the pouch, and 0.5 to 1.0 ml of physiological saline was injected while simultaneously aspirating with the first syringe, thus washing the pouch lumen and allowing the mucus to be collected.

**Method of infection.** Between 14 to 19 days post-surgery, the eight animals were randomly divided into two groups and infected. One group was infected intranasally by dripping 0.1 ml of a 1:100 dilution of live virus into the nostrils. The other group received the same amount of live virus injected into the tracheal pouch lumen. This was done between 4 and 5 p.m. on day 0.

**Viral assay.** Beginning on day 1 postinfection (between 10 a.m. and 12 noon on first day), nasal wash fluid and aspirations taken from tracheal pouches

were obtained daily for 8 days. When available, 0.1 ml of each sample was immediately inoculated into the allantoic cavity of two 10-day-old embryonated eggs. Pouch aspiration samples of smaller volume were diluted to 0.2 ml prior to infection. Nasal washes were diluted with an equivalent amount of a penicillin (50,000 U/ml) and streptomycin (250 mg/ml) solution to prevent bacterial contamination. Allantoic fluid was harvested from each egg after 48 h of incubation at 37 C and separated into two samples, one of which was frozen at -70 C. The unfrozen sample was assayed for the presence of virus by hemagglutination at a dilution of 1:2. For all samples showing no growth, the remaining sample was thawed and inoculated into a second pair of eggs in the event that the amount of virus present in the original inoculation was insufficient for detection by HA. The blind-passage eggs were incubated, harvested, and assayed in the same manner as for the original eggs. In several instances the isolated virus was shown to be similar to the infecting virus by serological identification (HI).

## RESULTS

Twenty-four hours prior to infection, two nasally infected animals and three pouch-infected animals were injected intraperitoneally with 20 ml of antiserum. All animals were bled immediately before inoculation and 14 days postinoculation. Nasal wash and pouch aspiration samples were taken daily for 8 days after infection. Table 1 shows the virus isolation results from nasal wash and pouch samples as well as the serum HI determinations.

From Table 1, it is clear that virus can be recovered from both the nasal passage and the tracheal pouch irrespective of the site of original infection; thus, one can conclude that the virus is disseminated from the pouch to the respiratory tract and vice versa. The duration of virus shedding from the nasal passage varies from 3 to 7 days, whereas shedding from the pouch varies from 1 to 5 days. The shedding from the nasal passage is always equal to or longer than that from the pouch. It is also clear that the presence of pre-existing, passively administered serum antibody, at HI titers 1:8 to 1:32 in the ferret, did not inhibit this dissemination. The passive administration of antibody, although not preventing dissemination of influenza virus, did inhibit the subsequent humoral antibody response.

## DISCUSSION

One of the first questions raised by the results presented here pertains to the mode of virus dissemination. Could it be via the blood, the lymphatics, or an artifact due to skin contamination? The latter seems highly unlikely since the pouch was a closed space, sterile equipment was used to obtain samples, and prior to aspira-

TABLE 1. *Virus dissemination between nose and pouch*

Route of infection	Serum HI titer (day 0)	Sample	Virus shedding by days <sup>a</sup>								Serum HI titer (day 14)	
			1	2	3	4	5	6	7	8		
No antibody												
Nose	<8	N <sup>b</sup>	+	+	+	+	+	+	+	+	-	2,048
		P	+	+	-	-	-	-	-	-	-	
Pouch	<8	N	+	+	+	+	-	-	-	-	-	1,024
		P	+	+	+	-	-	-	-	-	-	
Pouch	<8	N	+	+	+	+	+	+	+	+	-	1,024
		P	+	+	+	+	+	-	-	-	-	
Passive antibody												
Nose	8	N	+	+	+	+	+	+	+	-	-	128
		P	+	+	-	-	-	-	-	-	-	
Nose	32	N	+	+	+	+	+	+	+	+	-	16
		P	+	+	+	+	+	-	-	-	-	
Nose	16	N	+	+	+	+	+	-	-	-	-	32
		P	+	+	+	+	-	-	-	-	-	
Pouch	32	N	+	+	+	-	-	-	-	-	-	<8
		P	+	+	+	-	-	-	-	-	-	
Pouch	16	N	+	+	+	+	-	-	-	-	-	<8
		P	+	-	-	-	-	-	-	-	-	

<sup>a</sup> +, Virus isolated from sample; -, no virus isolated from sample.

<sup>b</sup> N, Nasal wash sample; P, pouch sample.

tion the skin was carefully washed with soap to destroy any possible influenza virus on its surface. There are no data available to invoke or rule out lymphatic dissemination, but since the blood supply to the pouch is left largely intact in the operation, it would seem that the lymphatics may also be partly preserved and that lymphatic dissemination is therefore possible. However, hematogenous dissemination seems the most likely mode. Although viremia has not been observed in the ferret (21, 22), more intensive studies in man have periodically led to detection of virus in blood, both in fatal cases (9, 11, 15) and during the incubation period of ordinary influenza (10, 13, 23). Since the disease in ferrets is similar to that in man (19), it seems likely that an intensive search during the early phase of infection (presumably within 24 h) would demonstrate such a viremia, since virus dissemination was detected in all animals within 24 h of infection. Even if viremia is undetected, hematogenous spread still would be possible, since one or a few virus particles arriving at the epithelium in the course of several hours may be sufficient for infection but would rarely be detected as a viremia. The hematogenous dissemination hypothesis is consistent with the observations of Basarab and Smith (2), who administered live influenza virus intravenously or intracardially and subsequently isolated virus from the nasal mucosa of six of the 10 ferrets.

The question as to mode of dissemination has taken on increased meaning since the appearance of reports suggesting a relationship between maternal influenza and both subsequent leukemia in the child (3) as well as increased pregnancy wastage (7). These associations could be due to indirect effects of the maternal infection or might result from infection of the fetus itself, as has been reported in mice (19). If direct infection is the source, then hematogenous spread is very likely the mechanism. The inability of antibody to inhibit the dissemination, demonstrated in this study, augurs poorly for antibody-mediated protection of such fetuses. It remains to be determined whether cell-mediated immunity might prevent such dissemination.

Although antibody did not inhibit dissemination, it did suppress the humoral antibody response to the infection. This has been observed in mice by Loosli et al. (12) and in our own more recent work (20), and is true irrespective of whether the pouch or the nasal passage is the site of infection. A more detailed study of these latter findings will be presented in another study.

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