Ferrets as a Transmission Model for Influenza: Sequence Changes in *HA1* of Type A (H3N2) Virus

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Ferrets were used as an animal model to study whether controlled transmission of type A influenza is similar to human transmission when sequence changes in *HA1* are used as the outcome. Ferrets were infected initially with A/Sydney/5/97 (H3N2) or A/LA/1/87 (H3N2) intranasally, and transmission chains were established by housing infected ferrets with noninfected ferrets with no influenza antibody titer against the infecting virus. Ferrets infected with A/Sydney were seronegative for A/Sydney and A/LA; ferrets infected with A/LA were seronegative for A/LA but had hemagglutination inhibition titers against A/Sydney. Titers of naturally transmitted influenza were higher than those after direct intranasal infection, but lymphocyte counts from nasal washes diminished with transmission. Ferrets infected with A/LA had 2 amino acid differences in *HA1* after transmission through 5 ferret cohorts, but those infected with A/Sydney had none. The results show the value of the ferret model. A/LA resembled the transmission of influenza in humans when under antibody pressure.

The tremendous impact of influenza virus infections on public health and the reduced productivity due to days lost from work are widely recognized [1]. Epidemics vary in severity from year to year and from community to community, and transmission patterns are still poorly understood. Much work has been done on the retrospective or historical evolution of all 8 genes of the influenza virus [2–15]; sequence variation arising from continued laboratory passage of influenza virus has been documented [16, 17]. However, the rate, extent, and pattern of influenza sequence evolution during human-to-human transmission are not understood. We know that influenza isolates from persons in the same community during the same outbreak show great sequence diversity. Influenza is caused by an RNA virus, which, as such, has a high mutation rate [18–20]. Sequence changes are a characteristic of an epidemic that has not been evaluated in an animal model. Use of a ferret model could allow controlled serial transmission, which is impossible to reproduce in a complex human epidemic. Should sequence changes be identified, the value of the ferret model to study other characteristics of human transmission would be established.

We used the ferret as an animal model for study of influenza because ferrets respond to influenza similarly to humans, with febrile illness, and are susceptible to human influenza. The influenza virus attaches via an α-2, 6 glycosidic linkage to the sialic acid of both ferret and human respiratory epithelial cells but attaches via the α-2, 3 linkage of sialic acid in avian and equine epithelial cells [21]. Ferrets also provide an opportunity to study a completely controlled population in which to observe the interplay of transmission with infection, illness, and sequence variation. Although a mouse model has been used [22, 23] to study influenza's epidemic course, its application to human influenza is limited, because the mouse is not a natural host.

The *HA1* sequence of the surface protein coded for by the hemagglutinin gene contains the most variable regions of the influenza genome. Bush et al. [4] reported 18 hypervariable codons in the human *HA1* gene. The gene has been studied well, and its sequence changes documented, in human populations. Thus, we monitored *HA1* throughout the transmission chain.

Animals, Materials, and Methods

Virus. We obtained the A/LA/1/87 (H3N2) virus from the laboratory of H. F. Maassab (University of Michigan, Ann Arbor). He isolated the virus in 1987, from a throat swab obtained from the Centers for Disease Control, in chicken kidney cells from specific pathogen–free animals (SPAFAS). Ferrets in this experiment were infected with nasal wash from ferrets infected with A/LA/1/87 CK1 SPAFAS Eggs 1 (SE1) 39°C SE6 35°C (10/12/99). The A/Sydney/5/97 (H3N2) virus was a gift from Aviron Laboratories. The pas-

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Animal experimentation guidelines of the University of Michigan Medical School Institutional Review Board were followed during these experiments.

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sage used to infect ferrets in this experiment was A/Sydney/5/97 SE2 35°C.

Ferrets. We used 26 6-week-old male ferrets, weighing 0.5 kg, from Marshall Farms. All tested seronegative against A/LA/1/87 (H3N2), and 13 were seronegative for A/Sydney/5/97 (H3N2). The other 13 had hemagglutination inhibition titers against A/Sydney, the circulating virus, of 5–20. All ferrets were treated daily for 3 days with 300,000 U of combination penicillin (Durapen, 0.1 mL; BioWhittaker).

Intranasal (inl) infection. The ferrets were lightly anesthetized with ether and were inoculated inl with 1 mL of the virus (0.5 mL per nostril). Two ferrets were infected with 1 mL of 1.5×10^6 pfu/mL A/Sydney/5/97 (H3N2). All ferrets used for the A/Sydney transmission chain were seronegative for A/Sydney. Two ferrets were infected with nasal wash from ferrets infected with A/LA/1/87 (H3N2). The nasal wash titered 1.4×10^5 pfu/mL. All ferrets used for the A/LA transmission chain were seronegative for A/LA but had hemagglutination inhibition titers of 5–20 against A/Sydney.

Transmission. Either 3 or 2 noninfected ferrets with no influenza antibody titer against the infecting virus (noninfected ferrets) were housed in a cage with 1 of the 2 ferrets infected inl with A/Sydney. The first of each group of these noninfected ferrets to develop a fever or to shed virus was removed and was caged with 2 more noninfected ferrets (4 ferrets per cage: 2 infected and 2 noninfected). The first of the 2 new noninfected ferrets to shed virus was then moved to a new cage with 2 more noninfected ferrets (1 infected and 2 noninfected). This continued until 5 cohorts were infected. The ferrets infected inl were called cohort 1; the noninfected ferrets housed with them were cohort 2. The same procedure was used with the 2 ferrets infected with the A/LA/1/87 nasal wash. Ferrets were moved as soon as they shed virus, to ensure the chain of transmission. Although the first infected ferret was used in the chain of transmission, all noninfected ferrets were monitored for shedding of virus and for temperature.

Temperatures. Rectal temperatures were taken 3 times a day (9 A.M., 12:30 P.M., and 4 P.M.) for 14 days, beginning the afternoon after infection. All temperatures were analyzed for fever by calculating the number of standard deviations from a normal average temperature of 38.6°C (obtained by averaging 72 normal ferret temperatures). A temperature of ⩾39.3°C (3 SD above the average temperature) was considered to be a fever. Earlier publications have used 2 SD as an indication of fever [24].

Nasal washes. Nasal washes with Tom Cat catheters (Vetpo) were done daily at 2 P.M. for 12 days on unanesthetized ferrets. In all, 3 mL of PBS was inserted inl, and an average ± SD volume of 2.4 ± 0.18 mL was expelled from the ferrets' nostrils and was collected in sterile urine collection cups. Nasal washes were tested for viral shedding by using the Directigen A rapid diagnostic test (Becton Dickinson) and were then placed on MDCK cells, to determine virus plaque-forming units per milliliter.

Nasal wash lymphocyte counts. Slides were prepared by using 25 μL of nasal wash per slide. Slides were air dried, fixed with methanol, and stained with standard Wright stain. For each nasal wash sample, lymphocytes from 20 fields were counted and averaged.

Human clinical influenza isolates. Four A/Sydney-like isolates from the 1998–1999 influenza season and 4 from the 1999–2000 season were taken from surveillance studies of the University of Michigan student body, to represent 2 successive outbreaks in a confined community of adults. Isolates were selected from the beginning, middle, and end of the outbreaks.

Sequencing. RNA from amplified nasal wash viruses and from the 8 human clinical influenza isolates was extracted by a guanidine-phenol extraction method and then was reverse transcribed with avian myeloblastosis virus reverse transcriptase (30 U). The product was used in the polymerase chain reaction (PCR) to amplify the cDNA, as described elsewhere [25]. The *HA1* of the hemagglutinin gene was amplified with primers HA3.009 (5′-CCCGGAAATGACAACAGCACAGC-3′) and HA3.975R (5′-CTCTGGTACATTCCGCATCCC-3′). PCR products were purified in agarose, and the amplified DNA was extracted from the gel by using PCR columns (Promega). Amplified hemagglutinin *(HA)* fragments were sequenced by using an automatic sequencer (model 377; ABI). The *HA1* sequence of each virus isolated from ferret nasal wash was compared with that of the wild-type (WT) A/Sydney/5/97 or the WT A/Los Angeles/1/87 infecting virus. The *HA1* sequence of each clinical isolate was compared with the *HA1* sequence of WT A/Sydney/5/97.

Results

Establishment of transmission chains between ferrets. Tables 1 (A/Sydney/5/97) and 2 (A/LA/1/87) document the ease of natural transmission of both viruses in ferrets. Although only the data from the ferrets infected first (donors) are shown, every noninfected ferret became infected. Because ferrets shed virus before they exhibit signs of illness, the Directigen rapid test proved to be the most reliable indicator of infection. Although the A/Sydney virus transmitted more quickly than the A/LA virus and achieved higher peak titers, the titer of the initial inoculum of A/Sydney was a log higher than that of the A/LA virus. However, the A/LA virus was partially ferret adapted, and ferrets exposed to A/LA had immune pressure from previous exposure to A/Sydney.

Nasal wash virus titers. Nasal wash titers from infected ferrets indicated that virus is shed from ferrets infected via transmission for 4–5 days, as found in inl ferret infection. Nasal wash titers after infection by virus transmission did not diminish as transmission continued. In fact, the highest titers were in ferrets infected by transmission, in cohorts 3 and 4, for both viruses.

Table 1. Donor ferrets infected with influenza A/Sydney/5/97 (H3N2): chain of transmission.

Cohort	Infection route	Day virus first present	Peak virus titer (day)	Peak temperature (day)	Day ferret moved
1	Inoculated inl	1	2.4×10^4 (4)	39.9°C (1)	0
2	Transmission	2	4.3×10^4 (3)	40.8°C (7)	3
3	Transmission	4	0.2×10^5 (6)	39.4°C (8)	5
4	Transmission	7	0.7×10^5 (11)	39.05°C (8)	7
5	Transmission	8	8.0×10^4 (10)	39.6°C (9)	NA

NOTE. Day 1 is first day after infection; all other days follow sequentially. Data reported are from the first ferret in each cohort to shed virus. All ferrets used were seronegative against A/Sydney/5/97 and against A/LA/1/89. A/Sydney/5/97 was a wild-type virus obtained from Aviron Laboratories. inl, intranasally; NA, not applicable.

Table 2. Donor ferrets infected with influenza A/LA/1/87 (H3N2): chain of transmission.

Cohort	Infection route	Day virus first present	Peak virus titer (day)	Peak temperature (day)	Day ferret moved
1	Inoculated inl	1	9.5×10^3 (1)	39.8°C (2)	0
2	Transmission	3	4.9×10^4 (5)	39.4°C (4)	3
3	Transmission	5	9.5×10^4 (6)	39.4°C (6)	6
4	Transmission	9	3.0×10^4 (9)	39.4°C (7)	9
5	Transmission	0	7.0×10^4 (12)	39.4°C (11)	NA

NOTE. Day 1 is first day after infection; all other days follow sequentially. Data reported are from the first ferret in each cohort to shed virus. All ferrets used were seronegative against A/LA/1/89 but had low hemagglutinin titers against A/Sydney/5/97. Inoculum was virus in nasal wash obtained from ferrets infected with wild-type A/LA/1/89. inl, intranasally; NA, not applicable.

Ferret temperatures. The appearance of fever, defined as 3 SD (≥39.3°C) above the average normal temperature, was more closely linked to viral shedding among ferrets infected with A/LA than among those infected with A/Sydney. One A/Sydney donor ferret did not exhibit fever even at 2 SD, although he eventually shed a high virus titer in nasal wash. Ferrets infected with A/Sydney attained peak temperatures of 39.1°C–40.8°C, whereas ferrets infected with A/LA had closely grouped peak temperatures of 39.4°C–39.8°C. Twelve of 13 ferrets exposed to A/Sydney and 12 of 13 ferrets exposed to A/LA exhibited fever (data not shown), if 3 SD is used to define a fever. If we define fever as 2 SD above normal temperature, 12 of 13 ferrets exposed to A/Sydney had fever, as did all 13 ferrets exposed to A/LA. Toms et al. [26] reported that a fall in virus titer followed the appearance of fever, but we did not observe this correlation of fever with virus titer. The first day of virus titer did not correlate with the first day of fever, especially for ferrets infected with the A/Sydney virus. For those infected with A/LA, fever usually appeared 1 day after the appearance of virus titer.

Lymphocytes in nasal wash. Influenza virus is internalized into antigen-presenting cells (APCs), and influenza antigens are displayed on the surface of the APCs, where they are presented to T helper and cytotoxic T cells [27]. The first lymphocytes to appear are the T helper cells and the cytotoxic T cells, followed by B cells ≥1 week later. The T cell component of the lymphocyte population escalates first, several days after infection [27]. Activated T cells are responsible for the eventual clearance of the virus [28, 29]. The day of the peak lymphocyte count lagged behind the first measurable shedding of virus in the nasal wash by 3–5 days for both viruses. Both viruses elicited a similar peak lymphocyte response in inl infected animals. However, the number of lymphocytes elicited in ferrets infected by transmission was considerably lower and appeared to diminish as transmission continued (data not shown). The last cohort infected was not followed up long enough to establish a lymphocyte response. The average lymphocyte response among the A/Sydney ferrets infected by transmission was 32, versus 51 for A/Sydney ferrets infected inl; the average lymphocyte response among A/LA ferrets infected by transmission or inl was 19 or 51 lymphocytes/μL of nasal wash, respectively.

HA1 *sequence from human isolates.* To document the sequence heterogeneity found in the influenza genome during successive epidemics in 1 locality, we compared the *HA1* sequence of 8 clinical A/Sydney-like isolates from the University of Michigan Student Health Service (1998–1999 season [n = 4] and 1999–2000 season [n = 4]) with the *HA1* sequence of WT A/Sydney/5/97. All 8 isolates differed in nucleotide sequence and in amino acid sequence, even though they were isolated from the same student population (see table 3). Of 12 silent variants in year 1, none was found in all 4 subjects that year. Of 14 variants coding for amino acid change, 6 were found in all 4 year 1 subjects. In year 2, 2 of 7 silent variants and 11 of 15 amino acid–changing variants were found in all 4 subjects. There were 12 cases of silent variation in year 1; only 1 of these was found in all 4 subjects in the second year. In contrast, there were 14 cases of an amino acid coding variant, 8 of which became fixed in year 2. Nine of the 12 silent variants disappeared in year 2, indicating that amino acid changes had become fixed in the population from selective pressures.

We found that aa 142 (present in all 8 isolates) and aa 144 (present in a single 1998–1999 isolate) fell into antigenic site A. Amino acids 192 (present in 1 1998–1999 isolate and 4 1999–2000 isolates) and 194 (present in all 8 isolates) fell into antigenic site B (data not shown).

The *HA1* of A/Sydney has 987 nucleotides. There were 49 nucleotide changes from A/Sydney and 34 amino acid changes from A/Sydney in the 4 *HA1* genes during 1998–1999. (These are differences from the base, not from within a population year.) This translates to a mutation rate of 1.24×10^{-2} mutations per nucleotide per outbreak for *HA1*. Thus, we observed variation across individuals from the same community in *HA* nucleotide sequences.

HA1 *sequence changes in ferrets.* After 5 transmissions in ferrets exposed to A/LA, we saw 2 *HA1* nucleotide changes,

Table 3. *HA1* sequence changes from wild-type influenza A/Sydney/5/97 in 4 clinical isolates from 1998–1999 (year 2 of A/Sydney as infecting virus) and 4 clinical isolates from 1999–2000 (year 3 of A/Sydney as infecting virus).

Variants	Isolates	
	1998–1999	1999–2000
Nucleotide variants within population	27	15
Nucleotide variants all different from A/Sydney	6	13
Amino acid variants within population	10	7
Amino acid variants all different from A/Sydney	6	11

NOTE. Data are no. of variants. Clinical isolates were obtained from the same student population. Variants within the population are a summation of all differences seen among the 4 clinical isolates (none were present in all 4). Variants all different from A/Sydney summarize the differences seen in all 4 clinical isolates different from A/Sydney.

Table 4. *HA1* sequence changes in 2 transmission chains of ferrets infected with influenza A/Sydney/5/97 or influenza A/LA/1/87 virus.

Virus, cohort	Infection route	Day	*HA1* amino acid nos.	Amino acids
A/Sydney			138, 186	Ala, Ser
1	Inoculated inl	1	138, 186	Ala, Ser
4	Transmission	8	138, 186	Ala, Ser
5	Transmission	11	138, 186	Ala, Ser
A/LA			138, 186	Ala, Ile
1	Inoculated inl	2	138, 186	X, Ser
4	Transmission	9	138, 186	Thr, Ser
5	Transmission	12	138, 186	Thr, Ser

NOTE. RNA was isolated from a positive nasal wash, and *HA1* was sequenced from a polymerase chain reaction product derived from the RNA isolated. inl, intranasally.

both of which coded for amino acid changes, in viruses isolated from nasal wash samples (table 4). These changes were beginning to appear in the A/LA virus extracted from the nasal wash of the cohort 1 ferret (a mixture of alanine and threonine at aa position 138 and all serine at aa position 186) and were unequivocally threonine and serine in the A/LA virus extracted from the cohort 4 ferret. Both the WT A/Sydney and the WT A/LA viruses coded for alanine at aa position 138, as did all the human isolates. The ferret isolates from those infected with A/LA mutated to threonine at aa position 138, whereas the human isolates (infected with A/Sydney) had a mutation at aa 137. Amino acid 138 was in antigenic loop A, and aa 186 was in antigenic loop B. The A/Sydney/5/97 *HA1* had serine at aa 186, to which the A/LA virus mutated in vivo. All human isolates retained serine at aa 186. By using the mutation rate for the *HA* observed in laboratory-passaged tissue culture of 1.35×10^{-5} mutations per nucleotide per generation (M.L.H.'s unpublished data), one would expect only 0.3 mutations to arise in 5 passages or 25 generations. In the noninfected ferrets exposed to A/Sydney, we saw no mutations in the *HA1* extracted from nasal wash samples.

Discussion

The rate of antigenic change and the molecular mechanisms driving that change in a human influenza epidemic are imperfectly understood. From earlier studies, we know that the sequences of the *HA* and neuraminidase genes vary from person to person, even within the same community during the same season. Some mutations, however, become fixed during an epidemic. To document this, the *HA1* sequence of 8 clinical isolates from the University of Michigan Student Health Service (4 from the 1998–1999 season, the second year that A/Sydney circulated, and 4 from the 1999–2000 season, the third year that A/Sydney circulated) were compared with the *HA1* sequence of WT A/Sydney/5/97. All 8 isolates differed in nucleotide sequence and in amino acid sequence, even though they were isolated from the same student population. We decided, therefore, to sequence *HA1* changes that occur after natural transmission, to help in determining the value of a ferret model in evaluating transmission of influenza in a human outbreak.

The study demonstrated that ferrets provide an excellent model for study of influenza transmission. The experiments proved that H3N2 viruses could be serially transmitted naturally from infected ferret to noninfected ferret simply by housing them together. Every noninfected ferret in the experiment became infected, even the ferrets under immune pressure, those with titers to A/Sydney/5/97 exposed to A/LA/1/87. It became apparent during the experiment that a rapid antigen detection test of nasal wash samples was superior to the appearance of fever as a way of determining virus infection. In these experiments, both A/LA/1/87 and A/Sydney/5/97 were serially transmitted through 5 cohorts of ferrets in 11 days, 1 of which was under antibody pressure similar to that in human transmission.

Although the ferrets cleared the virus ~6 days after both modes of infection, ferrets achieved a higher virus titer in nasal wash samples after infection by natural transmission than after infection by inl inoculation. The A/Sydney virus infected ferrets by transmission in 1–3 days, whereas the A/LA virus required 1–4 days for transmission. This could be the result of the protective titer the ferrets infected with A/LA had against A/Sydney, or it could reflect the lower initial titer used to infect.

One of the more intriguing findings of this transmission study was the lower induction of lymphocytes in the nasal wash after natural transmission. Cytotoxic T cells clear the virus from the respiratory tract of ferrets [28, 29], and virus-induced suppression of systemic cell-mediated immunity is a well-recognized phenomenon [30, 31]. Influenza is immunosuppressive. Some have described diminished lymphocyte transformation during influenza infection [32, 33]; others have suggested that cellular immunity is transiently impaired by influenza [34]. Our data suggest that lymphocyte counts in the nasal wash diminish as transmission proceeds. It is not clear whether a lower lymphocyte population is induced after natural versus inl infection or whether the lymphocyte population will continue to diminish with further transmissions. It is tempting to speculate that continually passaged influenza virus lowers the T cell response, thus damaging the response of the immune system to viral attack; however, the exploration of mechanisms will await validation of the conclusion. We can say that the peak lymphocyte response in the nasal wash follows the appearance of virus by 3–5 days, and, after lymphocyte appearance, the virus is cleared.

The variation observed across individuals from the same community in *HA* nucleotide sequences suggests antibody pressure in human transmission. In ferrets under immune pressure, influenza virus mutates to evade that pressure, just as it does in human transmission in which immune pressure is also present. After 5 transmissions in ferrets, we saw no sequence changes in the *HA1* extracted from the nasal wash of noninfected ferrets that were experimentally infected with A/Sydney. However, there were 2 nucleotide changes in the nasal wash of ferrets exhibiting immune pressure and infected with A/LA, both of which coded for amino

acid changes located in the hypervariable regions of *HA1*. These regions are involved in evading the immune response (table 4). Both mutations (at aa positions 138 and 186) seen in the nasal wash of ferrets infected with A/LA/1/89 were included in the 18 hypervariable codons in the human influenza *HA1* gene involved in antigenic selection [4].

The ferret model proved to be useful for evaluating transmission in a controlled situation. The virus attaches in a way similar to that among humans and results in clinical illness and further transmission. The antigen changes occurring under antibody pressure are similar to those in human outbreaks. Thus, this model would be appropriate not only to study further development of viral sequence diversity resulting in antigenic change, but also for study of the effect of vaccines and antivirals on virus infection acquired by the natural route.

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