Local and Systemic Immunity to Influenza Infections in Ferrets

W. HENRY BARBER AND PARKER A. SMALL, JR.

Department of Immunology and Medical Microbiology, University of Florida, College of Medicine, Gainesville, Florida 32610

To establish whether immunity to influenza infection in the ferret is local or systemic, two sites of challenge were utilized: the nose and the anatomically isolated tracheal pouch. Infection of either site did not spread to the other site, and challenge of either site resulted in seroconversion by 13 days. Simultaneous challenge of both sites 21 days after the primary infection revealed that prior infection of the pouch prevented subsequent reinfection of the pouch, but not infection of the nose. Thus, systemic immunity did not prevent the initiation of nasal influenza infection in the ferret. However, the duration of virus shedding from the nose was reduced to half of that seen when ferrets were infected for the first time, showing that the prior pouch infection did lead to a more rapid recovery from the subsequent nasal infection. Passively administered anti-influenza antibody did not prevent or modify the nasal infection, but it did prevent the pouch infection. This is consistent with the observation that an initial infection of the nose prevented pouch infection upon challenge 21 days later. The prior nasal infection also prevented the subsequent nasal infection. These data suggest that immunity to acquisition of influenza infection in the ferret is a local phenomenon, whereas recovery from active infection is influenced by systemic immune mechansims.

The host defense mechanisms responsible for protection from influenza infections in the human remain largely unknown. Some investigators have suggested that serum antibody is responsible for protection in the human, (28) while others have observed that influenza infection is not prevented by high levels of serum antibody (20). Several investigators believe that serum antibody correlates with, but does not cause, protection (16). There is also a question of the role of local antibody in immunity to influenza. Several workers (1, 23, 27) have shown that natural infection with respiratory viruses stimulates the production of immunoglobulin A antibodies in secretions and leads to resistance to reinfection, but as Burns and Allison (7) point out this does not necessarily imply that immunoglobulin A is responsible for the resistance. While it has been postulated that a vaccine's ability to stimulate nasal secretory antibody is the deciding factor in protection of humans against influenza infection, and some field trials of local immunization gave promising results (17, 32, 33), there are contrary data. Another trial of local immunization (10), as well as attempts to correlate secretory immunoglobulin A antibody with protection (9), was unsuccessful. It has also been proposed that cell-mediated immunity might be important in protection, since patients with hypogammaglobulinemia are not prone to more frequent or severe viral infections (12).

In contrast to the conflicting ideas and confusing data derived from human studies, there seems to be agreement that serum antibody is protective against influenza infection in chickens (2, 24) and in mice (19, 31). This apparent species-dependent difference in the host defense mechanism responsible for protection against influenza seems to result from a difference in the organs and tissues infected and not from a difference in immune mechanisms. Ramphal et al. (Fed. Proc. 37:1559, 1978) have shown that serum antibody does not prevent influenzal tracheitis in the mouse, but does prevent viral pneumonia. The discrepancies between the human studies and animal studies, therefore, probably relate to the fact that the immune studies of the mouse dealt with viral pneumonia, while the human studies dealt with tracheobronchitis.

Influenza infection of the ferret has been studied extensively and resembles the disease in the human (21). Hence, the ferret is a good animal model. Recent work has shown that passively administered antibody will not prevent initiation of nasal infection of the ferret. While passive antibody does suppress subsequent active syn-

thesis of serum antibody, it does not interfere with recovery. The study suggested that serum antibody was irrelevant to prevention of influenza and recovery from influenza in the ferret (26). The development of a surgical procedure, whereby an anatomically isolated segment of trachea is constructed, has enabled us to further explore the role of local and systemic immunity in prevention of influenza and recovery from influenza. Animals with tracheal pouches provide two separate sites, nose and pouch, for both infection and sample collection. Thus, it is possible to demonstrate that infection of the pouch does not subsequently induce immunity in the nose. This suggests that systemic immunity is not responsible for prevention of influenza in the ferret.

MATERIALS AND METHODS

Animals. Mature male ferrets were obtained from Marshall Research Animals, Inc., North Rose, N.Y., and housed in individual cages under conditions which prevent cross-infection. All ferrets were shown to have hemagglutination inhibition (HI) titers to A/PR/8 of less than 8 before use in the experiment. Tracheal pouches were surgically constructed as described in detail elsewhere (4). Briefly, the trachea was transected at two sites about 12 tracheal rings apart. The middle, or pouch, segment was displaced laterally, and the caudal end was closed with two sutures while the cephalad end was sutured to a piece of silastic tubing (ca. 2 to 3 cm long) which contained a recessed multipuncture seal in its distal end. The continuity of the trachea was reestablished by anastomosing the cephalad and caudal ends. The skin was closed so that the tube was subcutaneous.

Obtaining samples. Ferrets were anesthetized with 50 mg of Ketaset (Ketamine hydrochloride, Bristol Laboratories) per kg to obtain all samples. Blood was drawn by cardiac puncture. Nasal wash samples were obtained as described elsewhere (3) by slowly dripping phosphate-buffered saline into the animal's nose until he sneezed the material into a collection vessel. Pouch samples were obtained as described elsewhere (4) by inserting a 16-gauge needle through the skin and through the recessed plug of the silastic tubing. A polyethelene catheter was then inserted through the needle into the pouch, and the viscous fluid was aspirated with a tuberculin syringe. Both nasal wash and pouch samples were put on ice until they could be frozen at −85°C and held until analyzed.

Virus. The influenza virus used was A/PR/8/34 (HON1). A large stock was obtained by injecting virus into the allantoic cavity of 10-day-old embryonated chicken eggs which were then incubated for 3 days at 36°C, at which time allantoic fluid was harvested, pooled, and stored at −85°C in 1-ml portions. The allantoic fluid had a chicken erythrocyte hemagglutination titer of 1,280 and contained $10^{7.2}$ 50% egg infectious doses per ml (EID$_{50}$). When infecting ferrets, 0.1 ml of a 1:10 dilution containing $10^{5.2}$ EID$_{50}$ or 100 ferret 50% infectious doses was either dripped into the nose

of the anesthetized ferret or introduced through a catheter into the tracheal pouch.

Virus assays. Nasal wash or tracheal pouch samples were diluted with an equal volume of a phosphate-buffered saline solution containing 50,000 U of penicillin per ml and 250 mg of streptomycin per ml. About 0.1 ml of this mixture was injected into the allantoic cavity of 10-day embryonated chicken eggs and incubated for 3 days at 36°C. The allantoic fluid was then harvested, and a 1:10 dilution was assayed for hemagglutinating activity as previously described (2). If the sample was positive and sufficient material was available, serial 10-fold dilutions were injected in triplicate and the EID$_{50}$ was calculated by the method of Reed and Muench (22).

HI assay. HI assays were performed with the A/PR/8/34 as previously described (3) by using a microtiter kit and disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) according to the method of Sever (25). Sera used for HI assays were first absorbed with Kaolin and chick erythrocytes and heated at 56°C for 30 min.

Antisera. Serum for passive immunization was obtained from a number of ferrets convalescing from A/PR/8 infection. The pooled serum had an HI titer of 512. The normal ferret serum was from uninfected ferrets, and the pool had an HI titer of <8.

RESULTS

Experimental design. Two to three weeks before the first influenza virus challenge, tracheal pouches were constructed in 18 ferrets. Two days before virus challenge, eight of these, designated the PAb group, were given 40 ml of ferret antiserum intraperitoneally (20 ml in the morning and 20 ml in the evening). Eight other ferrets were given equal volumes of normal ferret serum (NFS group), and two control animals were given no prechallenge serum. On day 0 four animals from both the PAb and the NFS groups were infected via the pouch, and four were infected intranasally with influenza virus (A/PR/8). Twenty-one days after the primary challenge, all animals were rechallenged with the same strain of influenza virus simultaneously via both the nose and the pouch. The two control animals, not previously infected, were similarly challenged in both sites on day 21. Nasal washes and pouch secretions were collected on alternate days, stored at −85°C, and later assayed for influenza virus. Blood was drawn on days 0, 13, and 36 for serum antibody (HI) determinations.

Although the experiment on all 18 ferrets was carried out simultaneously, for clarity of presentation the description of the results will be divided into five parts, one for each of the five groups: (i) controls; (ii) passive antibody recipients originally challenged nasally; (iii) passive antibody recipients originally challenged via the pouch; (iv) normal serum recipients originally

challenged nasally; and (v) normal serum recipients originally challenged via the pouch.

Control animals. Table 1 summarizes the data for the two control animals challenged simultaneously in both the pouch and the nose on day 21. Both animals had no detectable antibody before challenge, but had developed high titers by 15 days after challenge (day 36). Both animals were shown to shed virus for 5 days from their noses. Animal 7A also shed for 5 days from his pouch, but 7B shed for only 3 days from his pouch. One and three days postinfection there was far more virus in the pouch secretions than in the nasal samples.

Animals receiving antisera challenged via the nose. Table 2 summarizes the data for the four ferrets which received antiserum on day −2 and were initially challenged via the nose. The day 0 HI titers represent the passive antibody. All four animals shed virus from their nares for 5 to 7 days after the initial challenge.

Upon rechallenge on day 21 via both the nare and the pouch, trace amounts of virus were detected in the nasal washes of three of the four ferrets on day 1 but not on subsequent days. No virus was detected in the pouch fluids. Although the virus shedding was minimal, there was a significant (greater than twofold) rise in antibody titer for two of the four animals, including 6B, which shed no detectable virus.

TABLE 1. *Virus and antibody titers for control animals challenged on day 21 via both pouch and nose*

Animal no.	Sampling site	Titer at day:					
		13	22	24	26	28	36
7A	Nose	<8^a	<1.8^b	5.3	+^{c}	0^d	2,048^a
	Pouch		≥6.7	5.7	≤2.2	0	
7B	Nose	<8	2.8	3.3	+	0	4,096
	Pouch		6.7	6.4	0	0	
Geometric mean							
Antibody		<8					2,900
Nose			<2.3	4.3	+		
Pouch			≥6.7	6.0	≤1.1		

^{a} HI titer.
^{b} Log$_{10}$ EID$_{50}$ per ml of virus recovered.
^{c} Virus recovered, but titration of amount of virus not performed.
^{d} No virus detected.

TABLE 2. *Virus and antibody titers for animals who received antibody and were challenged intranasally on day 0 and rechallenged on day 21 in both sites*

Animal no.	Sampling site	Titer at day:										
		0	1	3	5	7	9	13	22	24	26	36
6A	Nose	128^a	<2^b	+^{c}	3.7	<2		256^a	<2	0^d	0	512^a
	Pouch		0	0	0	0	0		0	0	0	
6B	Nose	256	4.4	+	3.5	0		64	0	0	0	256
	Pouch		0	0	0	0	0		0	0	0	
6C	Nose	512	3.0	+	5.0	≤2		128	≤2	0	0	2,048
	Pouch		0	0	0	0	0		0	0	0	
6D	Nose	256	3.7	+	4.5	<2		256	<2	0	0	512
	Pouch		0	0	0	0	0		0	0	0	
Geometric mean												
Antibody		256						152				610
Nose			<3.0	+	4.2	<2			<2	0	0	

^{a,b,c,d} See footnotes to Table 1.

Animals receiving antisera challenged via the pouch. Table 3 summarizes the data for the four ferrets which received antiserum on day −2 and were challenged via the pouch on day 0. The day 0 HI titers are comparable to those in Table 2 and represent the passively administered antibody. In marked contrast to Table 2, all four ferrets shed no virus from either the pouch or the nose. The HI titers fell to low levels by day 13, suggesting a half-life of the antibody of about 4 days.

On day 21 both the nose and the pouch were challenged. Both sites shed virus. The nose shed amounts comparable to those shed by the controls (Table 1) and the initial infections shown in Table 2. The duration of shedding was also similar to that shown in Table 1 and 2. The lack of shedding by 6H on day 22 is presumably an artifact of sample collection, storage, or assay. The infection stimulated a rise in HI titers comparable to that observed in virgin animals infected with influenza (26).

Animals receiving normal sera challenged intranasally. Table 4 summarizes the data for the four ferrets which received normal serum but no antibody and were challenged via the nose on day 0. All four shed virus from the nose for 5 to 7 days in amounts similar to other nasally infected animals. There was no evidence of any spread of the infection from the nose to the pouch, with the one exception of animal 6L, wherein a small amount of virus was detected in the pouch sample on day 7. We believe this one

observation is an artifact. The HI antibody titer increase was also similar to other primary infections. On rechallenge at day 21 of the nose and the pouch, both sites were immune, as judged by both a lack of virus shedding and a lack of significant antibody increase (greater than twofold).

Animals receiving normal sera challenged via the pouch. Table 5 summarizes the data from the four ferrets which received normal sera and were challenged via the pouch on day 0. All four shed virus from the pouch for 5 to 9 days. The duration and amount of virus shedding was equal to or perhaps greater than that seen in previously discussed pouch infections (Tables 1 and 3). The antibody HI rise was comparable in two of the four ferrets to that seen in previous primary infections, but reduced in the other two (6O and 6S).

On rechallenge of both the nose and the pouch on day 21, all four ferrets shed virus from the nose, but not from the pouch. Three of the four animals showed significant (fourfold or greater) rises in antibody titers during the reinfection period. The amount of virus shed from the nose on day 22, 1 day after challenge, was similar to day 1 shedding from the nose seen in the previous experiments (Tables 1 to 4), but by day 3 the amount of virus shed was decreased, and by day 5 there was no detectable virus.

Virus shedding. The virus shedding pattern for virgin ferrets is depicted in Fig. 1A and is characterized by peak virus titers 3 to 5 days

TABLE 3. *Virus and antibody titers for animals who received antibody and were challenged via the tracheal pouch on day 0 and rechallenged on day 21 in both sites*[a]

Animal no.	Sampling site	Titer at day:										
		0	1	3	5	7	9	13	22	24	26	36
6D	Nose	256ᵃ	0ᵇ	0	0	0		16ᵃ	2.0ᶜ	5.2	4.2	1,024ᵃ
	Pouch		0	0	0	0	0		≥7.2	0	0	
6F	Nose	256	0	0	0	0		32	3.6	4.5	3.0	512
	Pouch		0	0	0	0	0		6.0	3.0	3.5	
6G	Nose	128	0	0	0	0		32	3.6	4.2	3.3	4,096
	Pouch		0	0	0	0	0		4	5.7	0	
6H	Nose	256	0	0	0	0		8	3.5	<2	2.2	512
	Pouch		0	0	0	0	0		0	4.7	0	
Geometric mean												
Antibody		215						19				1,024
Nose									3.2	<4.7	3.2	
Pouch									≥4.3	3.4	0.9	

ᵃ,ᵇ,ᶜ See footnotes *a, d,* and *b,* respectively, to Table 1.

TABLE 4. *Virus and antibody titers for animals who received normal ferret serum and were challenged via the nose on day 0 and rechallenged on day 21 in both sites*

Animal no.	Sampling site	Titer at day:										
		0	1	3	5	7	9	13	22	24	26	36
6J	Nose	$<8^a$	2^b	$+^c$	5.5	0^d		$1,024^a$	0	0	0	256^a
	Pouch		0	0	0	0	0		0	0	0	
6M	Nose	<8	<2	3.2	4.5	0		8,192	0	0	0	512
	Pouch		0	0	0	0	0		0	0	0	
6K	Nose	<8	2	5.2	3.2	3.3		512	0	0	0	256
	Pouch		0	0	0	0	0		0	0	0	
6L	Nose	<8	4.4	<2	4.2	2.8		1,024	0	0	0	2,048
	Pouch		0	0	0	<3	0		0	0	0	
Geometric mean Antibody Nose		<8						1,450				512
Nose			<2.6	<3.5	4.6	1.5						

[a,b,c,d] See footnotes to Table 1.

TABLE 5. *Virus and antibody titers for animals who received normal serum and were challenged via the tracheal pouch on day 0 and rechallenged on day 21 in both sites*

Animal no.	Sampling site	Titer at day:											
		0	1	3	5	7	9	13	22	24	26	28	36
6O	Nose	$<8^a$	0^b	0	0	0		128^a	3.2^c	≤3	0	0	$1,024^a$
	Pouch		≥7.9	3.8	5.5	$+^d$	≤2.2		0	0	0	0	
6P	Nose	<8	0	0	0	0		512	4.5	≤2	0	0	1,024
	Pouch		6.8	4.2	3.8	0	0		0	0	0	0	
6R	Nose	<8	0	0	0	0		1,024	3	4.4^e	0	0	16,500
	Pouch		6.5	3.5	5.2	0	0		0	0	0	0	
6S	Nose	<8	0	0	0	0		128	3.2	$+$	0	0	512
	Pouch		≥7.4	3.4	4.5	5.5	<3		0	0	0	0	
Geometric mean Antibody Pouch Nose		<8						300					1,720
Pouch			≥6.9	3.7	4.8								
Nose									3.5	<2.5			

[a,b,c,d] See footnotes *a, d, b,* and *c,* respectively, to Table 1.
[e] Day 23.

postinfection and virus shedding for about 1-week duration. Figure 1B depicts the virus shedding pattern of ferrets previously infected in the pouch and challenged 21 days later in the nose (Table 5). Figure 1B shows that the nose shed as much virus on day 1 ($10^{3.5}$ EID_{50}) as in primary nasal infections ($10^{3.0}$, $10^{3.2}$, and $10^{<2.6}$ EID_{50} shown in Tables 2, 3, and 4, respectively). On day 3 there was less virus than on day 1, and by day 5 no virus was detectable. Figure 1C depicts the lack of virus shedding characteristic of solidly immune ferrets (Table 4).
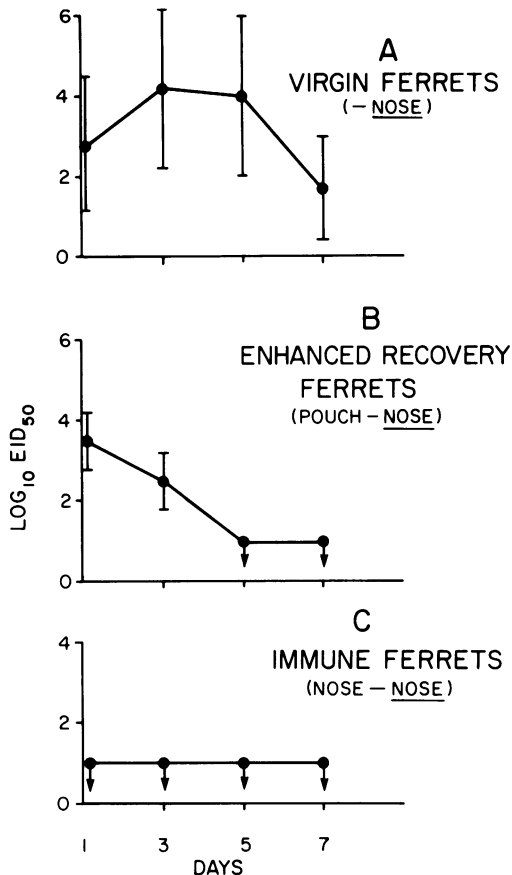
FIG. 1. Log_{10} EID_{50}/ml of influenza virus in nasal wash collected from ferrets 1, 3, 5, and 7 days after challenge. All animals were challenged on day 0. The symbol ↓ means that no virus was detected. A represents the nasal infection of the 14 previously uninfected ferrets. The data is taken from Tables 1, 2, 3, and 4. B represents the four ferrets infected in the pouch 21 days previously. The data is taken from Table 5. C represents the four animals which had been nasally infected 21 days previously. The data is taken from Table 4. Vertical bars represent the standard deviation.

DISCUSSION

Influenza virus dissemination does not occur between the tracheal pouch and the upper respiratory tract. This can be seen from the data presented in Tables 2, 4, and 5, which show that when influenza virus infects either the nose or the pouch, the virus replicates at that site but does not spread to the other site. This is not an artifact since the data in Tables 1 and 3 demonstrate that the virus can replicate simultaneously in both the nose and pouch if they are both challenged. The lack of virus dissemination is consistent with the inability to detect viremia

during influenza infection of ferrets (5) and humans (15). The observed lack of virus dissemination is contrary to data we previously published using a less refined surgical procedure (3). In the previous work, we were very careful to cut through only the mucosa and the submucosa of the dorsal aspect of the trachea in an attempt to preserve the blood supply. This necessitated maintaining the pouch in close proximity to the trachea, and we discovered subsequent to publication that re-epithelialization had taken place between the trachea and the pouch, thereby allowing direct spread of the virus. In the surgical technique used for our more recent work, we totally transected the trachea at both ends and we have not observed any re-epithelialization. The blood supply was adequate to maintain ciliary action for at least a year, and the histology remained normal for at least 6 months (4).

This study confirms earlier work demonstrating that passively administered antibody does not protect the upper respiratory tract of the ferret from influenza infection, even when the titers are raised to 1,024. Four days after infection, $10^{5.25}$ and $10^{4.5}$ EID_{50} of virus were recovered from these massively transfused animals (26). In the present study, the animals receiving passive antibody and challenged via the nose all became infected and shed virus for about the same duration in approximately the same amount as nasally infected animals without antibody. However, serum HI titers are not a reliable measure of infection under these circumstances since, as has been previously shown (3, 26), and again as observed in Table 2, passive antibody suppresses subsequent HI antibody production.

Although the passive antibody does not prevent nasal infection, it does prevent infections of the pouch (Table 3). No virus was shed from any of the four ferrets given passive antibody who were challenged via the pouch. It is uncertain whether this protective effect of serum antibody is indicative of biologically significant differences between tracheal epithelium and nasal epithelium or whether it is an artifact of the surgical procedure (e.g., pooling in the pouch of transudate containing serum antibody rather than having that transudate swept away by ciliary action). In either case, the nasal protection and susceptibility occurred in a physiologically unaltered nose and hence should represent normal events.

The most important conclusion from this work is that systemic immunity seems irrelevant to the prevention of the initiation of influenza infection in the ferret. If systemic immunity were important, one would have expected the animals previously infected in the pouch to be immune

in both the pouch and the nose. The one concern with this conclusion relates to whether an influenza infection of the pouch is an adequate stimulus for the systemic immune system. Two of the four ferrets (6O and 6S) had lower serum antibody titers than normally observed after nasal infection, but the other two (6P and 6R) were in the normal range, and yet all four animals were susceptible to nasal infection. Hence, these data strongly suggest that serum antibody is irrelevant to protection of ferrets from influenza. Since we have not measured cell-mediated immunity (CMI), we can not at this time rule out the possibility that a pouch infection does not stimulate CMI (even though it does stimulate serum antibody) and that systemic CMI is the host protective mechanism. However, this seems unlikely, especially since production of immunoglobulin G antibody to influenza virus has been shown to be T dependent in the mouse (14). The more likely explanation seems to be that local immunity is responsible for prevention of the initiation of influenza infection of the upper respiratory tract. Further work will be required to establish whether the local immunity is antibody or cell mediated.

The pattern and duration of virus shedding after nasal infection with influenza is shown in Fig. 1 to be markedly altered by a prior pouch infection. Hence, it appears that although the prior pouch infection has not produced immunity in the sense of preventing the infection, it has certainly enhanced the host recovery mechanism(s). The mechanism of enhanced recovery seen after a prior pouch infection appears to be a function of systemic immunity, although it is also possible that the prior pouch infection may have primed a local response. Since previous work suggested that serum antibody was irrelevant to recovery (26), CMI (or CMI-dependent interferon [14]) must be considered as a possible mechanism for recovery. The observation that guinea pigs previously immunized with influenza vaccine have been shown to give secondary CMI responses within 2 to 3 days of restimulation (13) is consistent with the rapid recovery being CMI dependent. The observation that suppression of CMI increases the duration of virus shedding in influenza-infected mice (29, 30) is also consistent with this concept. Nonspecific immunity must also be considered. Unpublished work from our lab has shown that infecting ferrets intranasally with A/Port Chalmers/1/73(H_3N_2) provides solid immunity 3 weeks later to the same virus given by the same route but not to A/PR/8 (H_0N_1) given intranasally. The A/PR/8 infects the ferret as shown by both specific antibody titer increases and virus isola-

tion from nasal washes, but the duration of shedding is reduced to less than 4 days. The enhanced recovery could either be explained by nonspecific immunity or by the decreased specificity of CMI observed with mouse T cells (11, 34). Thus, it is not possible to identify the mechanism responsible for recovery from infection. However, the recovery mechanism is not functional in ferret tracheal organ cultures since infected organ cultures shed virus for at least 60 days (8).

The observation that ferrets given passive antibody and infected via the nose were reinfected when challenged 21 days later in the nose (three of four shed virus, and the fourth ferret had a fourfold rise in antibody titers) but that they shed virus from the nose on just day 1 suggests that the passive antibody not only suppressed the serum antibody formation but also suppressed the local immunity. It also suggests that the passive antibody enhanced the recovery process. Since it has been reported (18) that passive antibody suppresses antibody formation but enhances CMI, this would be consistent with the possibility that the local immunity is antibody mediated and that the recovery is CMI dependent.

It appears that the mechanism for prevention of influenza infection in the ferret is a local phenomenon while the mechanism for recovery from infection is probably systemic and certainly different from that responsible for prevention of infection. Since the goal of most immunization programs is the prevention of illness, it is possible that this could be accomplished not only by prevention of the initial infection but also by greatly enhancing the recovery mechanism. Further studies of the specific effect of influenza immunization on these two separate phenomena may help explain some of the variability in the effectiveness of influenza immunization.

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