Influenza Infection in Ferrets: Role of Serum Antibody in Protection and Recovery

PARKER A. SMALL, JR.,* ROBERT H. WALDMAN, JEAN C. BRUNO, AND GEORGE E. GIFFORD

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

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The passive administration of ferret antiserum to Ao (H, N,1) influenza virus failed to protect the recipient ferrets from subsequent infection with homologous virus. This susceptibility to infection was observed even when the passively acquired serum hemagglutination inhibition (HI) titer was similar to peak convalescent titers. It is therefore concluded that serum antibody alone is probably not a major factor in the prevention of influenza infection. This does not rule out a possible role for serum antibody in prevention of illness. Subsequent to infection, ferrets that had received passive antisera failed to develop high levels of serum HI antibody. In fact, many had no detectable serum antibody (<1:8). These animals shed virus for periods of time quite similar to those of infected control animals, which did develop serum antibody. From these data it was concluded that detectable serum HI antibody does not play a significant role in the recovery of ferrets from influenza infection. Interferon was present in high concentrations in the secretions a few days prior to cessation of virus shedding, but it is not clear whether this was the cause of the recovery or merely a concomitant event. Twenty-one days after initial infection two-thirds of the ferrets that had received passive antibody and all control animals were immune to reinfection with the homologous influenza virus. Since the former group had little or no detectable serum HI antibody but most members were immune, there must be some other host mechanism to account for the immunity.

The mechanism(s) of immunity to influenza has been the subject of repeated study over the past 40 years but is still open to debate. Serum antibody, secretory antibody, and cell-mediated immunity have all been considered as being responsible for the prevention of disease and/or infection. Serum antibody seems to be the most commonly considered mediator of protection. In mice, serum hemagglutination inhibition (HI) antibody has clearly been shown to prevent death due to influenza (21, 36). In humans the data are not clear. It has been suggested that "immunity is dependent upon serum antibody levels first and foremost" (33), and Davenport has stated that vaccine-induced serum HI antibody titers of 1:32 or more (as measured with the epidemic strain) were associated with protection (17). However, other authors have observed a lack of correlation between serum antibody and protection (4, 24). In other studies comparing killed and live vaccines, killed vaccines stimulated higher serum HI antibody levels, while affording less protection than the live vaccine (3).

Respiratory secretion (immunoglobulin A) antibody has also been proposed as a mechanism of protection to influenza. Francis et al. (10) first suggested the importance of antibody in human secretion but presumed it was of humoral origin. de St. Groth and Donnelly (6, 7), from their studies in mice, suggested local synthesis. More recently some workers have observed protection as a result of local immunization (18, 27, 39). However, other trials of local immunization in animals (22, 28) and humans (8, 37), as well as attempts to correlate secretory immunoglobulin A antibody with protection (17), have been unsuccessful. Thus, the role of secretory antibody is also unresolved. It has been postulated that mechanisms other than those mediated by antibody, such as cell-mediated immunity, might be important in protection. The principal support for this idea is that patients with hypogammaglobulinemia do not seem to be particularly prone to more frequent and severe viral infection (12).

The mechanisms of recovery from influenza virus infection are also unknown. The postu-
lated mechanisms have involved antibody, interferon (2), and the destruction of all cells susceptible to the virus (11).

Thus the mechanism of immunity and, more specifically, the role of serum antibody in prevention of human influenza infection and/or illness is not clear. Furthermore, the direct test, i.e., the passive administration of antisemum as has been done in the mouse, is not a feasible approach in man. The ferret offers a practical alternative, since it develops influenza that is well characterized and closely resembles the human disease (11, 14, 15, 20, 23, 31, 34) rather than the fatal pneumonia characteristic of influenza in mice (21, 36). We therefore undertook the following studies on the effect of passive antibody on influenza infection in ferrets. The results suggest that detectable serum HI antibody is unlikely to be a central factor in either the prevention of or the recovery from influenza infection.

MATERIALS AND METHODS

Ferrets. Mature ferrets were obtained from Marshall Research Animals, Inc., North Rose, N.Y., and housed in individual cages in chemical hoods during the course of the experiment. Unpublished studies confirmed the original observations of Smith et al. (31) that passage of virus takes place from animal to animal when they are housed together. Ten or 12 animals housed in individual cages could, however, be kept in one hood with continuous flow of air without cross-infections. Males were used in all experiments except the high-dose passive antibody experiment, in which two females were used because they were smaller.

To obtain blood and/or nasal secretions, ferrets were first tranquilized by injecting ketamine hydrochloride (Parke-Davis) intramuscularly (80 mg/kg). Blood was drawn by cardiac puncture using a 22-gauge needle. Nasal secretions were obtained initially by inserting through the mouth a polyethylene tube with a 2-mm, 180° bend in the end, hooking the soft palate, and then flowing phosphate-buffered saline through the nasal passages out the nares into a beaker held under the animal's nose. Later in the study, nasal secretions were obtained as described by Potter et al. (25). Both methods yield similar material, but the latter is easier. Nasal secretions used for virus isolation studies were either stored at -70 C or inoculated directly into the allantoic cavity of 10-day-old embryonated chicken eggs. Nasal secretions used for antibody studies were stored at -30 C.

Virus. The influenza Ao (H3N1) virus used in this study has been previously described (1) and is a strain of PR8 that has been passed in embryonated eggs and tissue cultures numerous times. A large stock of this virus was grown for 3 days at 36 C in the allantoic cavity of 10-day-old embryonated eggs, pooled, and stored at -70 C in small aliquots. This stock virus had a chicken erythrocyte hemaggluti-

nation titer of 1:256 and contained 10^3.5 50% egg infectious doses per ml (EID_{50}), 10^6.5 50% tissue culture infectious doses per ml, and about 10^4.5 50% ferret infectious doses per ml. All ferrets challenged in this study were given 0.1 ml of a 1:100 dilution of this stock virus preparation intranasally. This dose has been 100% infective.

Assays. Virus was detected by inoculation of 0.1 ml of undiluted nasal wash fluid into 10-day embryonated eggs as previously described (1).

Hemagglutination and HI titers were performed with chicken erythrocytes by using a microtiter kit with disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) as described by Sever (29). Sera for use in HI tests were first adsorbed with kaolin and chicken erythrocytes as previously described (1).

Neutralizing activity of the secretions was determined by the hemadsorption-inhibition method (5) using rhesus monkey kidney cells infected with 10 to 100 50% tissue culture infectious doses of virus.

Interferon. Interferon was assayed by a yield reduction method on primary or secondary ferret kidney cells in screw-capped culture tubes (16 by 125 mm). Cells were treated for 18 h with 1 ml of a 1:5 dilution of serum or nasal wash. The nasal wash was previously dialyzed against 0.9 M NaCl, 0.01 M HCl (pH 2) for 24 h and then neutralized by dialysis against balanced salt solution, pH 7.4. The medium used was Eagle minimum essential medium with 10% fetal calf serum, containing penicillin and streptomycin at 250 U and 125 ugg per ml, respectively. Cultures were then washed two times and infected with vesicular stomatitis virus (Indiana serotype) at an input multiplicity of approximately five viruses per cell. After 1 h for virus adsorption, the residual virus was removed, and the cells were washed three times with medium and then incubated with 1 ml of medium for 24 h. Cultures were then frozen. The yield of virus was determined on L-cell monolayers using a plaque assay.

RESULTS

Normal controls. A series of normal ferrets was challenged with influenza virus intranasally and followed for clinical signs and development of serum HI antibody. Unfortunately, this virus did not produce an obvious temperature rise or marked lethargy, although ferrets did sneeze occasionally. Subsequent to infection they did, however, have a marked rise in HI antibody, which persisted in high titer to days 30 to 40 (Fig. 1). These animals and others were subsequently bled repeatedly and extensively to obtain a large pool of ferret anti-influenza antibody, which was used for passive immunization in the experiments discussed below.

To more clearly delineate the course of the infection, seven normal ferrets were infected and examined for: (i) virus shedding in secretions, (ii) serum antibody, (iii) secretory antibody, and (iv) interferon (Fig. 2). It can be seen
ROLE OF SERUM ANTIBODY IN INFLUENZA INFECTION

Fig. 1. Serum HI titers to influenza virus obtained at various times postinfection from a group of 10 ferrets not previously infected with influenza virus. Each dot represents a determination on an individual sample.

that the animals shed virus for 5 to 8 days. The two animals that died appeared to do so as a result of the cardiac punctures (cardiac tamponade at autopsy). The animals developed serum HI antibody levels comparable to those shown in Fig. 1. Nasal secretions showed no detectable HI activity, but did exhibit virus-neutralizing activity. The magnitude of the values is generally comparable to those obtained for ferrets by Shore et al. (30) when corrections are made for the $10^x$ concentration of secretions in their experiments. On the other hand, the activity observed in our experiments seems to be present a little earlier and to persist longer than theirs (peak values on day 7 with decline to undetectable levels by day 13). These differences are easily attributable to slight differences in the collection and assay techniques, especially since the secretory antibody levels were barely detectable and were not corrected for protein concentration (26).

Interferon was readily detectable in secretions, but almost absent from serum. The data from two animals are shown in Fig. 2 and similar observations were made on several other animals. These observations on interferon are somewhat at variance with those of Haff et al. (14), who found interferon in nasal tissues but not in nasal washings (or serum) of ferrets. They generally agree, however, with observations of influenza infection of humans, wherein Gesser and Dull (13) first reported interferon-like substances in nasal secretions and Jao et al. (16) subsequently repeated the observations and found interferon to be present in much higher concentrations in nasal secretions than in serum.

As was expected from previous work (9, 25, 31, 32), these seven animals were resistant to reinfection 3 to 5 weeks later, as shown by their lack of virus shedding subsequent to challenge, lack of serum HI antibody response, and lack of both secretory and serum (not shown in Fig. 2) interferon response.

Animals receiving passive ferret antibody to influenza virus. Figure 3 depicts the results of an experiment wherein each of five ferrets received intraperitoneally 20 ml of ferret antinfluenza antisera (HI titer, 1:512), followed 2.5 days later by intranasal challenge with live virus. All five animals had detectable serum HI antibody (due to the passive antibody) prior to challenge and all shed virus on days 2 and 3, the only days tested. The striking observation was the subsequent failure to develop serum HI antibody after initial infection. All five animals...
These animals had HI and the duration virus, HI serum before challenge with body responses. Two animals and ding nonshedders, and five all 20 ml of antisera with reinfection as judged both small, but 1 (Fig. had no animals, both virus shedders majority three of the five titers had more, on rechallenge at specifically received ferret whereas the control animals except that this symbols are similar above that the control and passive antibody

had titers of ≤1:8 on day 9 postinfection, whereas the control animals described above (Fig. 1 and 2) had titers of ≥1:256. Furthermore, on rechallenge at day 21, at which time all five animals had no detectable serum antibody, three of the five animals were resistant to reinfection as judged both by lack of virus shedding and lack of interferon response. The other two animals shed virus for less than 6 days and had no detectable interferon production. The majority of these animals, both virus shedders and nonshedders, subsequently developed small, but definite, serum and secretory antibody responses.

This experiment was repeated (Fig. 4) with 10 animals that were injected intraperitoneally with 20 ml of antisera with an HI titer of 1:256. These animals had HI titers of 1:32 or 1:64 before challenge with live virus. All 10 shed virus, and the duration was comparable to that of the control ferrets (Fig. 2). Once again the serum HI antibody diminished or disappeared, rather than rising to over 1:1,000 during the next 3 weeks as seen in the control group (Fig. 1 and 2). In this experiment secretory "antibody" was monitored more closely and appeared to be similar to that of the control animals depicted in Fig. 2. Although these values are quite variable, there appears to be a rise in virus-neutralizing activity. Therefore, the suppressive effect of the passive antisera had a much greater effect on serum antibody than on secretory neutralizing activity. This is consistent with local synthesis of antibody as suggested by Shore et al. (30). Interferon production was quite comparable to that of normal animals with influenza. Upon rechallenge of four of the animals, three did not shed virus and the fourth animal shed for only 3 days. None developed significant interferon in secretions or serum (not shown), and the subsequent antibody rise was less prominent than in the experiment shown in Fig. 3.

Although the duration of virus shedding was similar in the control and passive antibody

Fig. 3. Time course of several parameters after infection of ferrets that 2.5 days previously had passively received ferret antibody to challenge influenza virus. The symbols are similar to those of Fig. 2, except that (×) refers to the two animals that shed virus and (●) to the three that did not and (□) refers to assays wherein an end point was not reached. All the interferon assays were performed on nasal secretions.

Fig. 4. Time course of several parameters after infection of ferrets that 3 days previously had received passive ferret antibody to the challenge influenza virus. The symbols are identical to those used in Fig. 2.
groups discussed above, it is possible that the amount of virus shed could be quite different. To test this possibility, three ferrets that had never been previously infected were given passive antibody, challenged intranasally with live virus, and subsequently monitored quantitatively for virus shedding (by determining the number of EID_{50} doses in the nasal wash) and for serum HI antibody rise. Two animals receiving no passive antibody served as controls. Table 1 gives the results of this experiment. Once again passive antibody suppressed the serum HI response. Maximum virus shedding occurred between days 2 and 5 and was between 10^4.5 and 10^6.5 per ml of nasal wash for four of the five animals. Since nasal wash fluid is an unknown and variable dilution of the actual nasal secretions, exact comparisons were not possible. Evaluation of the dilution factor by measuring the protein concentration of the samples is not a satisfactory solution, since there was up to a fourfold variation among normal ferrets and a superimposed three-to-fivefold increase in protein concentration within a given animal as a result of infection (26). Within these limits there was no obvious suppression of virus shedding in the group given passive antibody.

To further investigate the role of passive antibody in protection from influenza, two female ferrets (=300 g each) were given a massive dose of ferret serum antibody to influenza virus. The antibody was prepared and concentrated by precipitation with 33% saturated ammonium sulfate so that it had an optical density at 280 nm (1 cm) of 21 (i.e., ≈16 mg/ml) and an HI titer of 1:1,024. Each ferret received 120 ml intraperitoneally during a 4-day period and had a serum titer of 1:1,024 just prior to challenge with the influenza virus. Both ferrets had virus in their nasal secretions 2, 4, and 6 days after challenge and hence fulfilled the definition of being infected. One animal shed 10^2.25 EID_{50} of virus whereas the other shed 10^2.75 on day 4. Two control animals that did not receive passive antibody shed 10^{5.25} and 10^{4.5} EID_{50} on day 4. The massive dose of passive antibody clearly did not protect either animal from infection and did not measurably suppress virus shedding in at least one of them.

**DISCUSSION**

Protection against infection. The experiments described in this paper suggest that serum antibody does not prevent influenza infection. Fifteen ferrets that were given passive ferret antibody to influenza in the form of whole serum had serum HI titers between 1:16 and 1:64 and were all readily infected when challenged with live virus intranasally. It can be argued that titers of this magnitude are inadequate to protect ferrets. We therefore gave massive doses of immunoglobulin to two ferrets and achieved passive in vivo HI titers of 1:1,024 (i.e., similar to peak postinfection titers in ferrets) just prior to challenge. This large amount of passive antibody did not protect the ferrets from infection. Since the antisera were produced in response to infection, we presume there was also a large amount of antineuraminidase antibody present, although we did not measure it. From these data it is tempting to conclude that serum HI and perhaps antineuraminidase antibody cannot, in and of itself, protect the ferret from influenza infection. There are, however, two possible objections to this conclusion. One is that the challenge dose was too large to demonstrate the effectiveness of the serum antibody. Although this is possible, it should be pointed out that the dose of virus was not sufficient to overcome the natural immunity present 21 days postinfection. The other is that the ammonium sulfate fractionation procedure used in the high-dose experiments removed a class or subclass of antibody that is protective. It should be emphasized that this data dose not rule out a possible role for serum antibody in the prevention of disease, as opposed to infection. Passive serum HI antibody has been clearly shown to prevent death of mice.

<table>
<thead>
<tr>
<th>Animal tested</th>
<th>HI titer (day 0)</th>
<th>Virus shed (log_{10} of EID_{50}/ml)</th>
<th>HI titer (day 14)</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;16</td>
<td>5.5</td>
<td>6.3</td>
</tr>
<tr>
<td>Passive antibody</td>
<td>256</td>
<td>&lt;3</td>
<td>4.5</td>
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<tr>
<td></td>
<td>256</td>
<td>4.25</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>128</td>
<td>&lt;3</td>
<td>2.5</td>
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*Each animal was infected intranasally with 0.1 ml of a 10^-5 dilution of influenza virus on day 0. Virus shedding refers to the amount of virus in nasal secretions obtained as described (26).*
when given prior to influenza infection (21, 35), yet it did not prevent infection (21). Challenge studies in humans have also shown that it is possible to reinfest three of four volunteers within less than a year while they still had demonstrable HI antibody but none of them became clinically ill during the second infection (24).

In addition to susceptibility to influenza infection in the presence of serum antibody, resistance in the absence of detectable serum antibody was also observed. All the ferrets given passive antibody prior to challenge had decreased convalescent serum HI antibody titers when compared with infected controls. Nine of these animals were challenged 21 days after their original infection and six were found to be immune, whereas three shed virus for 3 or 4 days but showed no significant interferon response. Thus, six were immune and the other three were partially immune, in spite of the fact that five of the nine had no detectable serum antibody and the other four had very low titers. We conclude that the immunity must be due to a host defense mechanism other than serum HI antibody.

Several host defense mechanisms might be suggested to explain the immunity present after infection with influenza virus: (i) secretory antibody-mediated immunity, (ii) cell-mediated immunity, or (iii) nonspecific resistance. Secretory antibody is certainly a possibility, but from our data and those of others (22, 28, 30) it is not possible to draw compelling conclusions. The secretions are greatly and variably diluted during collection and hence the titers represent the “tip of an unknown iceberg.” We hope that repeating the experiments using animals with tracheal pouches (1) will enable us to obtain tracheal secretions without dilution and thereby to be able to better evaluate the role of secretory antibody. Cell-mediated immunity, either local (38) or systemic, seems to be a possibility. The work of Liew and Parish (19) has shown that homologous or heterologous passive antibody can enhance cell-mediated immunity at the same time it inhibits antibody responses. Hence, there is no reason to rule out the possibility that the immunity present at 21 days in the immune suppressed animals might not be cell mediated. But more work is clearly indicated. Nonspecific factors, such as interferon or exhaustion of susceptible cells, remains to be considered.

Recovery from infection. As stated earlier, all of the animals that received passive antibody and were subsequently infected had markedly reduced serum HI titers to influenza and a few had no detectable serum antibody shortly after cessation of virus shedding. In spite of this apparent lack of serum antibody, these animals did not appear to be different from control infected animals in terms of duration of virus shedding or interferon response to the infection. The amount of virus shed may have been comparable or perhaps somewhat reduced in the passive antibody group. We therefore conclude that detectable serum HI antibody alone plays no role in ferrets’ recovery from influenza.

What is responsible for recovery from influenza? Neutralizing activity is clearly present in secretions of our normal and “suppressed” ferrets subsequent to infection, but not prior to the primary infection. In previous experiments with ferrets, Shore et al. (30) have shown virus-neutralizing activity of secretions obtained in early convalescence from influenza infection. As discussed above, our data on the levels of neutralizing activity in secretions are too variable to draw definite conclusions, but secretory antibody cannot be ruled out as being important in recovery. Interferon is present in secretions in high concentrations and at the right time to account for the cessation of virus shedding, but our data do not prove a causal relationship. They only show a temporal one. Another possible explanation is that the virus has destroyed all the susceptible cells and there are none left in which the virus can replicate. Francis and Stüart-Harris (11) showed that the virus destroys the respiratory epithelial cells and causes essentially complete desquamation of the ciliated columnar epithelium by day 4, and by day 6, when the animal is convalescent, its respiratory tract is lined with transitional epithelium. Since the virus replicates in ciliated epithelial tissue, it is certainly possible that the destruction of that tissue is a significant factor in determining the cessation of virus replication. Additional unknown factors or combinations cannot be excluded.

Suppression of antibody response. Observations similar to those reported here were made in the mouse influenza model by Loosli et al. (21) over 20 years ago and have largely lain fallow since. These investigators challenged mice, previously immunized in a variety of ways, with PR8 influenza virus and observed that passive antibody did not prevent virus shedding, although it did reduce the duration and amount of virus shed. HI serum antibody response to the infection was markedly reduced, so that 21 days after infection the passively immunized animals had no detectable antibody (<1:16) in marked contrast to other groups (e.g., 1:768). When these mice were rechallenged 21 days after the onset of the first
infection, more than one-half were resistant to between 10 and 1,000 50% lethal doses of influenza virus. These animals did develop serum antibody subsequent to the second challenge. The obvious similarity of the general design and results of their and our experiments suggests that the same basic phenomenon is operative in both mice and ferrets in spite of the different disease produced by influenza virus in these species (mice get a fetal pneumonia whereas ferrets get tracheobronchitis). They used rabbit antiseria in mice, whereas we used ferret antiseria; hence both heterologous and homologous antiseria seem able to induce the immune unresponsiveness.

In light of the work that has been done subsequent to that of Loosli et al. (21) in studying the effects of passive antibody on the immune response (see review by Uhr and Möller [35]), it is possible to draw analogies with the observations made using influenza virus, an immunogen which can replicate, and the literature using nonrepllicable immunogens. Clearly passive antibody can be immunosuppressive for antibody formation. The suppression of the host systemic antibody response to an infectious agent may prove to be a technique of considerable value in the study of other infectious diseases. In the experiments presented here, it has helped to delineate the role, or lack thereof, of detectable serum antibody in protection and recovery from infection with influenza virus. This phenomenon may also be important in infections of the newborn where the infant has received passive antibody transplacentally from its mother.

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LITERATURE CITED


