

Heterotypic Immunity to Influenza in Ferrets

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Heterotypic immunity to influenza virus in ferrets operated against heterotypic influenza viruses but not heterologous viruses. Contrary to prior reports, the protection conferred lasted for at least 18 months. This type of immunity limited virus shedding but did not prevent infection. These results suggest that this phenomenon could play a role in determining the severity of infections caused by type A influenza viruses in humans.

In the companion paper (9), we have shown that the heterotypic immunity described by Schulman and Kilbourne (6) is a critical factor in determining the severity of influenza infection. Heterotypic immunity limits the spread of virus in the respiratory tract and prevents lethal viral pneumonia. For these phenomena to be clinically significant, they must persist at least from one influenza season to another. McLaren and Potter (3), however, reported that heterotypic immunity in ferrets did not persist for more than 10 weeks. This limited duration would suggest that the phenomenon is of little clinical significance and would cast doubt on the hypothesis that heterotypic immunity might be the mechanism for the apparent increase in immunity to influenza in humans as they age. Consequently, this study was undertaken to determine the persistence of heterotypic immunity in ferrets.

MATERIALS AND METHODS

Animals. Adult male ferrets were obtained from Marshal Research Animals, Inc., North Rose, N.Y., and were housed in cages under conditions which prevent cross-infection (8). Animals received feed and water ad libitum.

Viruses. The viruses used, A/PR/8/34(H0N1), A/PC/73(H3N2), and B/Lee/40, were obtained from Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Stocks of all three viruses were raised in the allantoic cavities of chicken embryos.

Infection. Ferrets were anesthetized with 50 mg of ketamine hydrochloride per kg of body weight and infected with 0.1 ml of a dilution of virus in phosphate-buffered saline, pH 7.2, containing $10^{5.2}$ 50% egg infectious doses by dropwise instillation into the nose.

Virus isolation and titration. Virus was isolated by nasal wash, as described by Barber and Small (2). Phosphate-buffered saline was introduced into the nose until the animals sneezed the material into a collection vessel. All samples were separated into two equal portions and frozen at -80°C until they were assayed.

Virus was detected by injecting 0.1 ml of sample into the allantoic cavity of 10-day embryonated chicken eggs which had previously received 0.1 ml of antibiotic solution containing 250,000 U of penicillin per ml and 250 mg of streptomycin per ml. The eggs were incubated for 3 days at 36°C . The allantoic fluid was harvested and tested for hemagglutination in a method described by Allan et al. (1). If the sample was positive, serial 10-fold dilutions of the second portion were injected into eggs in triplicate, and the 50% egg infectious dose was calculated by the method of Reed and Muench (5).

Serum antibody titers. Blood was obtained from anesthetized ferrets by cardiac puncture. Sera used for hemagglutination inhibition assays were first adsorbed with kaolin and chick erythrocytes and heated at 56°C for 30 min (2). Hemagglutination and hemagglutination inhibition titers were performed with a microtiter kit, using disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) as described by Sever (7).

Statistical analysis. Viral titers and the course of virus shedding were compared by Student's *t*-test (4). For the purposes of statistical analysis, \log_{10} of undetectable amounts of virus was defined as zero.

Experimental design. Preinfection blood samples were taken by cardiac puncture. Serum antibody titers were measured to ensure that the ferrets had not previously been exposed to influenza or viruses. The ferrets were infected with one of three viruses: A/PR/8/34(H0N1), A/PC/73(H3N2), or B/Lee/40. Nasal wash samples were collected from all animals on days 1, 3, 5, 7, and 9. Twenty-one days postinfection, blood samples were drawn to determine convalescent titers. On day 21 or 12 to 18 months after the first infection, animals were challenged with homotypic, heterotypic, or heterologous virus. Nasal wash samples were collected on days 1, 3, 5, 7, and 9 after the challenge infection, and a blood sample was drawn 14 days after the challenge infection.

RESULTS

Short-term heterotypic immunity. In the first experiment, 18 ferrets were divided into two groups of nine. One group was infected with A/PC/73(H3N2), and the other was infected with

A/PR/8/34(H0N1). Twenty-one days later, each group of nine was divided into three subgroups of three. Of each group of nine, one subgroup was infected with each of the previously mentioned viruses, and one subgroup was used for another experiment. Thus, the antibody titers and pattern of virus shedding of each challenge virus were established in virgin ferrets and ferrets convalescent to both of the viruses. The hemagglutination-inhibiting antibody data (Table 1) demonstrate that the first infection, be it with either A/PC/73(H3N2) or PR/8/34(H0N1), induced specific antibody to the infecting virus but not to the other virus. One ferret did show a rise in titer to both viruses after the first infection, and the animal was dropped from the study as having been coinfecting. Rechallenge with the same virus produced no rise in antibody to either virus. Rechallenge with the heterotypic virus produced an increase in titer to the rechallenge virus, but not to the original virus.

The course of virus shedding in virgin and convalescent ferrets infected with H0N1 is shown in Fig. 1a. As expected, ferrets convalescent from an H0N1 infection were solidly immune to rechallenge with the homotypic virus as evidenced by the absence of virus shedding. There was no significant difference between the virus titers of the other groups on day 1 or 3 of the challenge infection. However, ferrets convalescent to H3N2 infection ceased shedding virus by day 5, whereas the virgin ferrets shed virus until day 7.

The course of virus shedding in convalescent ferrets challenged with H3N2 is shown in Fig. 1b. Previous infection with the homotypic virus afforded solid immunity. Ferrets convalescent to

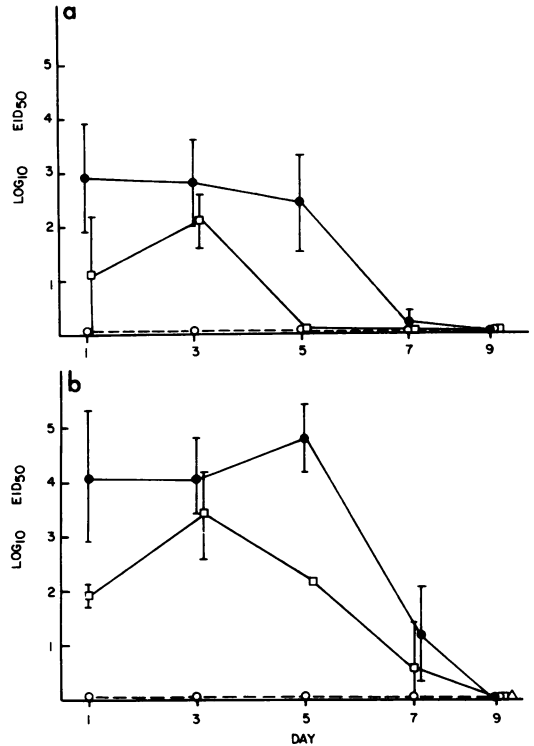


FIG. 1. (a) Comparison of A/PR/8/34(H0N1) virus shedding from virgin and convalescent ferrets. Nine virgin ferrets (●), three ferrets convalescent to A/PR/8/34(H0N1) (○), and three convalescent to A/PC/73(H3N2) (□) were used. Means and standard errors are presented. If no bar is shown, the standard error is contained within the point. Undetectable levels of virus are represented by log₁₀ = 0. (b) Comparison of A/PC/73(H3N2) virus shedding from virgin and convalescent ferrets. Nine virgin ferrets (●), three ferrets convalescent to A/PC/73(H3N2) (○), and two convalescent to A/PR/8/34(H0N1) (□) were used. Other details as in (a).

TABLE 1. Serum hemagglutination inhibition titers from ferrets before and after sequential infections with influenza^a

Infecting virus		Titer against:					
		Before first infection		After first infection		After second infection	
		A/PC	PR/8	A/PC	PR/8	A/PC	PR/8
A/PC	A/PC	10	<8	256	<8	256	<8
	PR/8	8	<8	161	<8	315	128
PR/8	PR/8	10	<8	<8	128	<8	128
	A/PC	8	<8	<8 ^b	128	256	51

^a Geometric means of the titers against A/PC/73(H3N2) and A/PR/8/34(H0N1) are given for each group.

^b One ferret showed a titer of 256 against A/PC/73 after a first infection with A/PR/8/34 and was excluded from the study as having been coinfecting.

H0N1 shed significantly less virus than virgin ferrets on days 1 and 5, and one of the two animals had stopped shedding virus by day 7.

Long-term heterotypic immunity. To determine how long heterotypic immunity might last, four ferrets that had been infected with A/PR/8/34(H0N1) 18 months previously were challenged with A/PC/73(H3N2) (Fig. 2a). The heterotypically immune ferrets shed significantly less virus ($P < 0.01$), except on day 1, and shed virus for a shorter time than did the virgin ferrets. In another experiment, five of seven ferrets showed decreased viral yields and duration of shedding when challenged with heterotypic virus 12 months after their first infection (data not shown). Four ferrets infected with B/

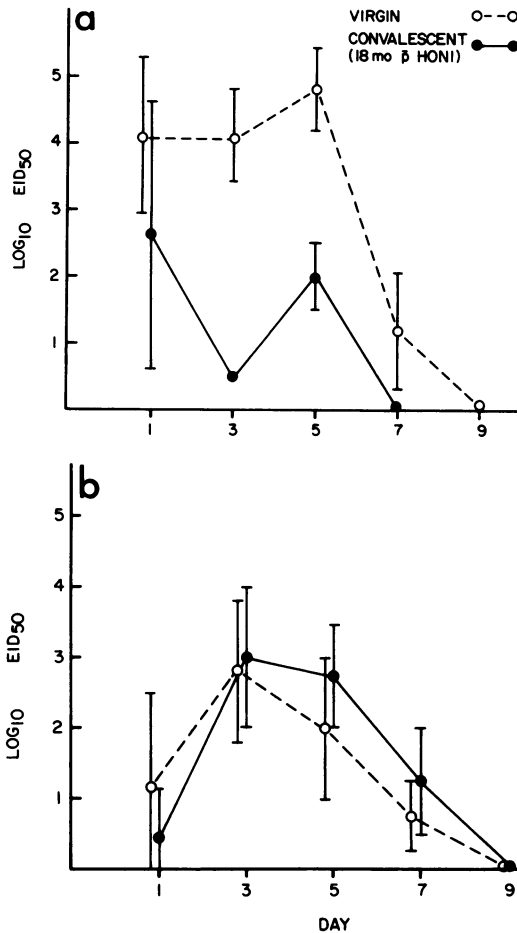


FIG. 2. (a) Comparison of A/PC/73(H3N2) virus shedding from nine virgin (○) and four heterotypically immune (●) ferrets. The virus shedding data from virgin ferrets are replotted from Fig. 1b. The heterotypically immune ferrets had been infected 18 months previously with A/PR/8/34(H0N1). Means and standard errors are presented. If no bar is shown the standard error is contained within the point. Undetectable levels of virus are represented by log₁₀ = 0. (b) Comparison of B/Lee/40 virus shedding from four virgin ferrets (●) and four ferrets convalescent to A/PC/73(H3N2) (○). Other details as in (a).

Lee/40 12 months after a first infection with A/PC/73(H3N2) showed virus shedding patterns comparable to those of virgin ferrets infected with B/Lee (Fig. 2b).

DISCUSSION

Our results demonstrate that heterotypically immune ferrets show a more rapid fall and earlier cessation of virus shedding than do virgin ferrets after infection with type A influenza virus. In most cases, the day 3 virus titers of virgin

and heterotypically immune ferrets were comparable. This was seen even when the day 1 virus titers were lower in heterotypically immune animals than in virgin animals. This pattern of virus shedding suggests enhanced recovery. The late onset of this heterotypic immune mechanism may indicate that a memory or recall phenomenon is occurring. This enhanced recovery may have been responsible for the increased survival of heterotypically immune mice reported by Schulman and Kilbourne (6). Enhanced recovery has also been reported to occur after challenge of convalescent ferrets with homotypic influenza virus at an isolated site (2).

McLaren and Potter (3) reported that heterotypic immunity as determined by day 1 and day 3 virus titer differences was a short-term phenomenon. In contrast, by examining virus shedding on days 5, 7, and 9 in addition to days 1 and 3, we have shown that heterotypic immunity, as demonstrated by enhanced recovery, persists for at least 18 months. We have also found that the enhanced recovery of convalescent ferrets challenged with homotypic virus at an isolated site persists for at least 18 months (R. A. Yetter, W. H. Barber, and P. A. Small, Jr., unpublished data).

Thus, this study shows that heterotypic immunity is not a short-lived phenomenon. An enhanced recovery mechanism that operates for at least 18 months after the first influenza infection could be of clinical significance by playing a subsequent role in the response of humans to influenza infections. It would be of value to know the mechanism responsible for this phenomenon so that its duration and effect could be evaluated in humans.

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LITERATURE CITED

- Allan, W. H., C. R. Madeley, and A. P. Kendall. 1971. Studies with avian influenza A viruses: cross protection experiments in chickens. *J. Gen. Virol.* 12:79-84.
- Barber, W. H., and P. A. Small, Jr. 1974. Dissemination of influenza virus between anatomically isolated sites in ferrets. *Infect. Immun.* 9:530-533.
- McLaren, C., and C. W. Potter. 1974. Immunity to influenza in ferrets. VII. Effect of previous infection with heterotypic and heterologous influenza viruses on the response of ferrets to inactivated influenza virus vaccines. *J. Hyg.* 72:91-100.
- Mendenhall, W. 1975. Introduction to probability and statistics, p. 214. Duxbury Press, North Scituate, Mass.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* 27:493-497.

6. **Schulman, J. L., and E. D. Kilbourne.** 1965. Induction of partial specific heterotypic immunity in mice by a single infection with influenza A virus. *J. Bacteriol.* **89**: 170-174.
7. **Sever, J. L.** 1962. Application of a microtechnique to viral serological investigations. *J. Immunol.* **88**:320-329.
8. **Small, P. A., Jr., R. H. Waldman, J. C. Bruno, and G. E. Gifford.** 1976. Influenza infection in ferrets: role of serum antibody in protection and recovery. *Infect. Immun.* **13**:417-424.
9. **Yetter, R. A., S. Lehrer, R. Ramphal, and P. A. Small, Jr.** 1980. Outcome of influenza infection: effect site of initial infection and heterotypic immunity. *Infect. Immun.* **29**:654-662.