Response of Ferrets and Monkeys to Intranasal Infection with Human, Equine and Avian Influenza Viruses

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SUMMARY

Rhesus monkeys and ferrets were exposed to intranasal inoculation of several strains of egg-adapted avian, equine and human influenza viruses and to strains of mouse-adapted equine influenza viruses. Local replication of virus and seroconversion were observed in the majority of these animals. However, clinical infection was observed only in ferrets.

RÉSUMÉ

Des furets et des singes rhesus ont été inoculés par la voie intranasale avec diverses souches de virus influenza aviaire, équine et humaine adaptées à l'embryon de poussins et avec des souches equines adaptées à la souris. Chez la majorité de ces animaux, les virus se sont repliqués dans les voies respiratoires supérieures et ont produit une séroconversion importante. Toutefois, l'infection clinique n'a été observée que chez le furet.

INTRODUCTION

The interrelationships between human and animal influenza viruses have long been studied, notably between swine and human influenza strains (1, 8, 11, 18, 26, 28). These studies have been intensified recently for four reasons: first, several A strains of influenza virus have been isolated from equine and avian spp. (23, 29, 33), one B strain from horses (7); second, that an A/Equine-2 isolate was obtained in the same locality as an antigenically related human influenza subtype 2 strain (19); third, that serological surveys and experimental studies have confirmed the antigenic relationships between some equine, avian and human influenza viruses (5, 10, 20, 21, 22, 25, 31, 32), and fourth, transmission studies in which man has been found susceptible to A-Equine-2 isolates (14, 15), ponies to human influenza (2-16) and dogs to human A2 and B strains of influenza viruses (30).

Information has been sought on the response of ferrets and monkeys to isolates of avian, equine and human strains of influenza since there has been little work by others in this area (3, 4, 13, 24).

MATERIALS AND METHODS

SUBJECTS

Healthy four to five pound rhesus (*Macaca mulatta*) monkeys and nearly mature ferrets were used in these experiments. These animals were kept isolated throughout the observation period. Each animal was bled before, and 14 and/or 28 days after inoculation. Signs of illness were recorded daily.

VIRUSES

The avian Myxovirus influenzae strains AA6/Turkey/Ont/3724/63, AA5/Turkey/Ont/6213/66, AA5/Turkey/Ont/7732/66 (17) as well as the Myxovirus parainfluenzae/Yucaipa/1959 (9) were kindly supplied by Dr. G. Lang from the Ontario Veterinary College, University of Guelph. The Myxovirus A/Equine-1/Prague/262/56 was supplied by Dr. J. T. Bryan from the University of Kentucky and the Human A2 variant Hong Kong virus was supplied by the Communicable Disease Center, Atlanta, Georgia. The A/Equine-2/Richelieu/63 was isolated by the authors in 1963 during a

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natural outbreak of equine influenza (19). All these strains have undergone several passages on chick embryo. Mouse-adapted A/Equine-1 and A/Equine-2 strains were also used in these experiments.

Inoculation of Animals

The ferrets under ether anaesthesia received 0.5 ml of virus suspension in each nostril. The monkeys, under nembutal and ether anaesthesia, received in each nostril 1.0 ml of virus suspension.

Virus Recovery

Nasal washings were collected 48 and 96 hours after infection. Five ml of antibiotic containing PBS were introduced into each nostril of the animals and approximately 5.0 to 7.0 ml of liquid were recovered in each case. The nasal washings were centrifuged at 2000 RPM for 30 minutes, then serial tenfold dilutions of the supernatants were titered in 11 day embryonated eggs, using five eggs per dilution. Eggs dying between two and six days after inoculation, as well as the embryos still alive 144 hours post inoculation, were examined for viral hemagglutinins.

Serological Techniques

The hemagglutination-inhibition (HI) tests were performed in plastic disposable trays, using the macrotiter method (6). Receptor-destroying enzyme of Vibrio cholerae was used to destroy non-specific inhibitors in the sera prior to HI tests (6).

Determinations of the Specificity of the Animal Responses

In the two series of infection trials with the different viruses, specificity of the serological response in individual animals was determined by the cross-HI test, using the stock virus strains as antigens.

TABLE I. Response of Ferrets and Monkeys to Various Strains of Myxoviruses Inoculated Intranasally

Virus Strains	Infecting Dose $log_{10}EID_{50}$/ml	Animal No.	Virus Isolated from Nasal Washings $log_{10}EID_{50}$/ml		HI Antibodies Days after Infection		
			48 hours	96 hours	0	14	28
Monkeys							
Parainfluenza/Yucaipa/59....	9.5	1	1.1	1.7	0	60	30[a]
		2	2.1	2.2	0	10	0
AA6/Turkey/Ont/3724/63....	8.5	3	3.9	3.2	0	0	30
		4	1.5	0.0	0	5	60
A2/Hong Kong/1/68.....	7.5	5	4.0	3.9	0	160	320
		6	0.9	1.5	0	160	320
A/Equi-1/Prague/56.....	4.2	7	1.7	2.0	0	30	30
		8	2.8	2.5	0	80	60
A/Equi-2/Richelieu/63	6.7	9	0.8	2.3	0	40	30
		10	1.0	1.2	0	20	0
AA5/Turkey/Ont/7732/66....	6.0	11	0.0	0.0	0	0	0
		12	0.0	0.0	0	80	120
Ferrets							
AA6/Turkey/Ont/3724/63....	8.5	1	4.2	6.5	0	160	
		2	4.5	4.0	0	240	
AA5/Turkey/Ont/7732/66....	7.8	3	3.7	4.7	0	160	
		4	2.5	1.8	0	240	
AA5/Turkey/Ont/6213/66....	8.2	5	4.5	4.4	0	120	
		6	5.2	4.7	0	480	

[a] Reciprocal of serum dilution.

In addition, in the second series of infection trials, the viruses isolated from the nasal washings were identified by the cross HI test, using standard antisera.

RESULTS

First Series of Infection Trials

Following intranasal inoculation of monkeys and ferrets with the virus strains indicated in Table I, evidence of virus replication was observed in all animals, except in the two monkeys inoculated with the avian strain AA5/Turkey/Ont/7732/66. HI antibodies were detectable in all the experimental animals, except in one monkey inoculated with the above mentioned strain. None of the monkeys showed evidence of clinical infection. However, all the ferrets inoculated showed some signs of rhinitis accompanied sometimes by sneezing and shivering. No appreciable rise in temperature was noted in monkeys or ferrets. Cross-HI test performed on individual sera demonstrated that these animals were infected by the inoculated viruses (Table II).

Second Series of Infection Trials

Four monkeys were inoculated with two mouse-adapted equine strains. Eight ferrets were inoculated with the same mouse-adapted equine strains, human A2/Hong Kong/59 strain and the avian paramyxovirus strain Yucaipa/1959. All the animals infected with the influenza viruses excreted virus 48 and 96 hours after inoculation,

TABLE II. Cross Inhibition-Hemagglutination Test with Convalescent Sera vs Stock Virus

Antisera	Animal No.	Viruses					
		Parainfluenza/Yucaipa/59	AA6/Turkey/Ont/3724/63	AA5/Turkey/Ont/7732/66	A/Equi-1/Prague/56	A/Equi-2/Richelieu/63	A2/Hong Kong/1/68
Yucaipa/1959	Monkey 1	0/20[a]	0/0	0/0	0/0	0/0	0/0
	" 2	0/0	0/0	0/0	0/0	0/0	0/0
AA6/3724/63	" 3	0/0	0/15	0/0	0/0	0/0	0/0
	" 4	0/0	0/20	0/0	0/0	0/0	0/0
AA5/7732/66	" 11	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
	" 12	0/0/0	0/0/0	0/80/120	0/0/0	0/0/0	0/0/0
A/Equi-1/Prague/56	" 7	0/0/0	0/0/0	0/0/0	0/30/30	0/0/0	0/0/0
	" 8	0/0/0	0/0/0	0/0/0	0/80/60	0/0/0	0/0/0
A/Equi-2/Richelieu/63	" 9	0/0/0	0/0/0	0/0/0	0/0/0	0/40/30	0/0/0
	" 10	0/0/0	0/0/0	0/0/0	0/0/0	0/20/0	0/0/0
A2/Hong Kong/1/68	" 5	0/0	0/0	0/0	0/0	0/0	0/480
	" 6	0/0	0/0	0/0	0/0	0/0	0/320
AA6/3724/63	Ferret 1	0/0	0/120	0/0	N.D.[b]	0/0	30/30
	2	0/0	0/240	0/0	N.D.	0/0	15/15
AA5/7732/66	" 3	0/0	0/0	0/240	N.D.	0/0	15/15
	" 4	0/0	0/0	40/480	N.D.	0/0	N.D.
AA5/6213/66	" 5	0/0	0/0	20/40	0/0	0/0	15/15
	" 6	0/0	0/0	0/160	0/0	0/0	15/15

[a]Reciprocal of serum dilution: Serum before infection/Serum two weeks after infection/Serum four weeks after infection.
[b]N.D. = Not done.

developed antibodies against the inoculated virus (Table III) and presented clinical reactions similar to those observed in the previous experiment. The sera of each animal reacted specifically in the cross HI test (Table IV).

Isolates from the nasal washings of each animal were found by the cross HI test to be identical to the inoculated viruses (Table V). Two ferrets inoculated with the mouse-adapted strain A/Equine-1/Prague/56 shed the inoculated virus strains 48 and 96 hours post infection and showed specific seroconversion, as shown in Table IV. However, these animals presented a significant rise of temperature on the seventh day post inoculation. The fever was followed by dyspnea, depression, heavy nasal discharge and the ferrets were moribund on day 14. At necropsy, discoloration of the liver and spleen and a purulent sinusitis were observed in both animals; in one of these, a hemorrhagic gastroenteritis was also observed. The turbinates of these ferrets were removed and a hemagglutinating agent was recovered in high concentration from the homogenates of these turbinates. No inhibition of the hemagglutination was observed when this agent was tested against standard antisera prepared with the egg-adapted myxovirus strain A2/Aichi/68, A/Equine-1/Prague/56 and A/Equine-2/Richelieu/63. Therefore we can assume that these ferrets were secondarily infected with another hemagglutinating agent. Work is underway to identify this agent. It is reported that low concentration of virus has been isolated from the turbinates of ferrets inoculated with the influenza virus strain A/PR8/34 up to 14 days after intranasal inoculation. However, these authors did not mention identification of the isolated viruses (12).

The two ferrets inoculated with the Myxovirus parainfluenzae Yucaipa/1959 did not excrete virus 48 and 96 hours after injection nor produce antibodies against that strain (Table III).

DISCUSSION

The data obtained in this study indicate that infection of ferrets and of non-human primates with egg-adapted strains of influenza viruses isolated from different animal species can be easily accomplished. Al-

TABLE III. Response of Ferrets and Monkeys to Various Strains of Myxoviruses Inoculated Intranasally

Virus Strains	Infecting Dose $log_{10}EID_{50}$/ml	Animal No.	Virus Isolation from Nasal Washings $log_{10}EID_{50}$/ml		HI Antibodies Days after Infection	
			48 hours	96 hours	0	14
Monkeys						
A/Equi-1/Prague/56 (M.A.)[a]	6.6	15	3.1	0.9	0	240[b]
		16	1.8	0.8	0	2560
A/Equi-2/Richelieu/63 (M.A.)	6.8	17	2.4	1.0	0	160
		18	1.0	1.7	0	480
Ferrets						
A/Equi-1/Prague/56 (M.A.)	6.6	9	4.2	2.7	0	320
		10	4.0	1.3	0	640
A/Equi-2/Richelieu/63 (M.A.)	6.8	7	5.0	2.3	0	320
		8	4.3	3.3	0	320
Parainfluenza/Yucaipa /59	9.1	11	0.0	0.0	0	0
		12	0.0	0.0	0	0
A2/Hong Kong/1/68	9.8	13	4.3	4.5	30	240
		14	3.0	5.2	40	240

[a](M.A.) = Mouse-adapted.
[b]Reciprocal of serum dilution.

TABLE IV. Cross Inhibition-Hemagglutination Test with Convalescent Sera vs Stock Viruses

Antisera	Animal No.	Viruses: A/Equi-1/Prague/56 Pre-infection	A/Equi-1/Prague/56 Post-infection	A/Equi-2/Rich./63 Pre-infection	A/Equi-2/Rich./63 Post-infection	Parainfluenza Yucaipa/59 Pre-infection	Parainfluenza Yucaipa/59 Post-infection	A_2/Aichi /68 Pre-infection	A_2/Aichi /68 Post-infection
A/Equi-1/Prague/56 (M.A.[a])	Monkey 15	0	240[b]	0	0	0	0	0	0
	" 16	0	2560	0	0	0	0	0	0
A/Equi-2/Rich. /63 (M.A.)	" 17	0	0	0	160	0	0	0	0
	" 18	0	0	0	480	0	0	0	0
A/Equi-1/Prague/56 (M.A.)	Ferret 9	0	320	0	0	0	0	40	60
	" 10	0	640	0	0	0	0	40	80
A/Equi-2/Rich. /63 (M.A.)	" 7	0	0	0	320	0	0	40	80
	" 8	0	0	0	320	0	0	60	80
Parainfluenza/ Yucaipa/59	" 11	0	0	10	10	0	0	60	80
	" 12	0	0	10	0	0	0	80	40
A2/Hong Kong /69	" 13	0	0	0	10	0	0	30	240
	" 14	0	0	0	0	0	0	40	240

[a](M.A.) = Mouse adapted
[b]Reciprocal of serum dilution

TABLE V. Cross Inhibition-Hemagglutination Test with Virus Isolates vs Standard Antisera

Virus Isolates from Animals Inoculated with	Animal No.	Standard Antisera: A2/Aichi/68	A2/Montreal /68	A/Equi-1/ Prague/56	A/Equi-2/ Richelieu/63
A/Equi-1/Prague/56 (M.A.)[a]	Monkey 15	0	0	480[b]	0
	" 16	0	0	960	0
A/Equi-2/Richelieu/63 (M.A.)	" 17	15	0	0	960
	" 18	15	0	0	960
A/Equi-1/Prague/56 (M.A.)	Ferret 9	0	0	1280	0
	" 10	0	0	960	0
A/Equi-2/Richelieu/63 (M.A.)	" 7	5	0	0	1280
	" 8	15	0	0	480
A2/Hong Kong/69	" 13	960	0	0	5
	" 14	960	0	0	10

[a](M.A.) = Mouse-adapted
[b]Reciprocal of serum dilution

though signs of infection in monkeys exposed to virus were essentially subclinical in nature, it appeared that these animals were nevertheless infected. Antibody production and/or virus replication have been obtained in each group of monkeys. Mouse-adapted strains of equine viruses appear to stimulate a higher production of circulating HI antibodies than the egg-adapted strains, although local virus replication was similar in both cases. As reported earlier (24), the susceptibility of monkeys to a mouse-adapted human influenza strain can be increased by subjecting the animals to experimentally altered conditions such as diet, cold or route of inoculation. Successful natural transmission to cage mates was recently reported (13). This indicates that a non-human primate may act either as a vector or as a host for human influenza viruses. Cage mate transmission and experimentally altered conditions were not undertaken in our study and it would be interesting to see if avian and equine influenza infections could be transmitted by similar techniques.

The results obtained in ferrets show that these animals are more susceptible than monkeys, except for the avian paramyxovirus strain Yucaipa/59. With this strain, no virus replication or antibody production occurred. All the other ferrets showed some signs of a mild clinical infection, except those ferrets inoculated with the mouse-adapted A/Equine-1/Prague strain which developed a severe illness on day 7. The nature of this particular event is under investigation. Most investigators have found that it is not unusual to find circuulating HI antibodies against human strains in normal ferrets and it is also interesting to note the relative susceptibility of ferrets to egg-adapted influenza viruses.

From the results reported in this paper and from those reported by other investigators, one can observe that the host barrier of influenza viruses can be readily overcome under experimental conditions and there is no reason why this could not happen naturally. These findings support the reported serological evidence that strains found in animals may have been responsible for human influenza in the past. They also suggest, along with other reported data, that human influenza viruses could be the origin of some animal influenzas.

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