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Experimental Production of Respiratory Tract Disease in Cebus Monkeys After Intratracheal or Intranasal Infection with Influenza A/Victoria/3/75 or Influenza A/New Jersey/76 Virus

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A total of 28 cebus monkeys were inoculated intratracheally or intranasally with 10^6 50% tissue culture infective doses of A/New Jersey/76 virus or 10^7 50% tissue culture infective doses of A/Victoria/75 virus, and 8 additional monkeys received sterile allantoic fluid. Each of the animals became infected as evidenced by a serological response and/or shedding of the virus. Of the 10 animals inoculated intratracheally with A/Victoria/75 virus, 8 developed a systemic illness, and pulmonary infiltration was detected by X-ray in 7 of the 8. Administration of A/New Jersey/76 virus intratracheally to 10 monkeys produced a mild systemic illness in 2 animals and an upper respiratory tract illness in 6, but no illness developed in the remaining 2 monkeys; none of the animals developed X-ray evidence of lower respiratory tract disease. Intranasal administration of either virus failed to induce any illness or produced, at most, mild illness confined to the upper respiratory tract. These studies demonstrate that cebus monkeys are susceptible to respiratory tract infection with influenza A viruses and that the development of pulmonary disease is reflected in the appearance of easily recognizable radiological changes.

In the adult human population influenza A virus is the most important primary viral pathogen of the lower respiratory tract, acting not only as a self-sufficient pathogen but also as a stimulus that initiates a sequence of events that lead to bacterial pneumonia (7, 17). As a result of the complex epidemiology of influenza A virus, new serotypes have emerged to which the population has little or no immunity. The interval between the recognition of a new, potentially pandemic virus and its dissemination throughout the world may be less than 6 months. During this interval, it would be highly desirable to be able to rapidly evaluate in a nonhuman primate anti-influenza A virus prophalactic and/or therapeutic modalities, such as the protective efficacy of candidate live and inactivated vaccine strains or the effectiveness of antiviral compounds against the new strain. Because of the growing shortages of nonhuman primates, it would be helpful if the evaluation would not require sacrifice of the animal. This paper describes the response of cebus monkeys to intratracheal (i.t.) or intranasal (i.n.) administration of two strains of influenza A virus of human origin, influenza A/Victoria/3/75 (H3N2) and influenza A/New Jersey/8/76 (Hsw1N1). The results indicate that the cebus monkey represents a useful nonhuman primate model for influenza A virus infection in that quantitative clinical, radiological, virological, and serological data can be obtained without sacrifice of the animal.

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MATERIALS AND METHODS

Viruses. The wild-type influenza A/Victoria/3/75 (H3N2) virus was isolated originally from a throat washing by Brian Ferry, Victoria, Australia. This same throat washing was subsequently sent to Alan Kendal, Center for Disease Control, Atlanta, Ga., where the virus was reisolated in specific pathogen-free, avian leukosis virus-free embryonated hens' eggs. In our laboratory the virus underwent one additional passage in the allantoic cavity of leukosis virus-free eggs.

The influenza A/New Jersey/8/76 (Hsw1N1) virus was isolated from throat swab material obtained at Fort Dix, N.J., by Alan Kendal. After isolation in embryonated eggs, (SPAFAS, Inc., Storrs, Conn.) the virus underwent a total of 11 passages in primary calf kidney cell cultures (Flow Laboratories, Rockville,

Md.), including double-plaque purification. A suspension was then prepared in the allantoic cavity of embryonated hens' eggs. The viral suspensions were tested for adventitious microbial agents by techniques previously described (12); no such agents were found.

Monkeys. Thirty-six seronegative cebus monkeys (*Cebus apella* and *Cebus albifrons*) were selected for use in these studies. Animals were equally divided between laboratory-raised juveniles and feral adults; all were in excellent health at the time of inoculation. Monkeys were maintained on a 24% Wayne Monkey Diet supplemented biweekly with apples, bread, and vitamin D_3 in the water.

Experimental protocol. Three separate studies were performed; the experimental design of each was identical. Animals were housed in air-flow-controlled isolation cages (10) after pairing according to weight. After a 1-week period of observation during which base line laboratory values for each animal were established, 28 monkeys were given a light general anesthetic (Vetalar, Parke-Davis & Co., Detroit, Mich.) and, while in a supine position, were inoculated either i.t. or i.n. with 1.0 ml of 10^6 50% tissue culture infective doses ($TCID_{50}$) of influenza A/New Jersey/76 or 10^7 $TCID_{50}$ of influenza A/Victoria/75 virus. i.t. administration was performed by direct laryngoscopy using the outer sheath of a 12-gauge intravenous catheter as the endotracheal tube. Supplemental anesthesia was provided the larger animals by intravenous pentobarbital; in each instance the cough reflex remained intact, thereby exposing both the lower and upper respiratory tract to the i.t. inoculum. An additional eight animals were anesthesized and inoculated with an equal volume of sterile allantoic fluid both i.n. and i.t. in an identical manner. After inoculation, animals were observed daily for signs of illness. Rectal temperature was monitored daily (same time each day to minimize the effect of diurnal variation) for the first 10 days after inoculation and also on days 14 and 21. Throat swabs were obtained daily and placed in veal infusion broth containing gentamicin, amphotericin, and 0.5% gelatin. Samples were then inoculated in 0.25-ml quantities into duplicate sets of primary rhesus monkey kidney (RMK) roller tube cultures for recovery of virus. Blood was collected for hematological and serological studies before administration of virus and 2, 6, and 21 days later. Chest X rays were obtained on a similar schedule and as warranted by clinical signs of respiratory tract disease. On day 4 to 6 after inoculation, at least one animal from each group was sacrificed and specimens of the respiratory tract were obtained for gross and histological examination.

Titration of inocula. Virus inocula were serially diluted and assayed for infectivity by hemadsorption in primary RMK roller tube cultures (Flow Laboratories).

Immunological procedures. The hemagglutination inhibition (HAI) test was performed in microtiter plates; 4 antigen units of each virus containing either the A/Victoria/75 (H3N2) or A/New Jersey/76 (Hsw1N1) hemagglutinin were used.

Laboratory studies. Complete blood cell counts were performed by Bionetics Laboratories (Rockville, Md.).

Criteria for designation of illness. The following categories of illness were defined to evaluate clinical response: (i) respiratory tract illness (RTI) present on a minimum of 2 consecutive days during which at least two of the following signs were present—rhinorrhea, sneezing, excessive lacrimination, and conjunctivitis; (ii) systemic illness consisting of the aforementioned criteria for RTI plus the presence of dyspnea, lethargy, depressed reaction to stimulation, or marked anorexia. The base line temperature range of individual monkeys was 37.0 to 40.4°C and varied in response to the handling of the animal and to changes in ambient temperature. For these reasons a temperature elevation in the monkeys was difficult to document.

RESULTS

Response to i.t. inoculation. After the i.t. administration of 10^7 $TCID_{50}$ of influenza A/Victoria/75 virus, each of 10 animals became infected, as evidenced by viral shedding and/or a serological response (Table 1). Virus was recovered from throat swabs as early as day 1 post-inoculation, and the average duration of shedding was 5.7 days (range 2 to 10 days). By day 3 to 5, each of the 10 animals manifested signs of illness; systemic illness was evident in 8, while 2 monkeys developed only signs of RTI. Systemic illness appeared to be maximal from day 4 to day 7, and the affected animals sat in the corner of their cage with arms hanging limply at their sides and exhibited a markedly depressed reaction to normal stimuli. Dyspnea was manifested by intercostal and supra-clavicular retractions, paradoxical abdominal respiration, and nasal flaring. By day 14 all animals had recovered fully except for mild rhinorrhea, which persisted as long as 21 days in three animals. A fourfold or greater serum HAI antibody rise was observed in each of the six monkeys tested.

i.t. inoculation with 10^6 $TCID_{50}$ of influenza A/New Jersey/76 virus infected each monkey and produced clinically detectable respiratory tract disease in 8 of 10 animals. Virus was recovered from only 1 of the 10 animals. In contrast to the response observed after infection with A/Victoria/75 virus, illness was generally mild and limited to the upper respiratory tract. The two animals that exhibited systemic signs of infection were less ill than those infected with the Victoria strain. A fourfold or greater rise in serum HAI antibody was observed in each of the six monkeys tested.

No signs of illness were detected in control animals inoculated both i.n. and i.t. with sterile allantoic fluid; the monkeys remained seronegative.

Response to i.n. inoculation. A/Victoria/75 virus was recovered from each of the four animals inoculated (Table 1). Onset of virus shedding (day 1) and average duration of shedding

TABLE 1. *Response of seronegative (serum HAI antibody ≤1:4) cebus monkeys to i.t. or i.n. administration of wild-type influenza A virus*

Virus	$TCID_{50}$	Route of administration	No. of animals	Virus isolation			Serum HAI antibody response		% Infected as evidenced by shedding and/or serological response	Clinical response		
				No. who shed virus	Initial day of shedding (range)	Avg duration of shedding (range)	No. with fourfold or greater rise[a]	Avg fold increase		No illness	RTI[b]	Systemic illness[c]
A/Victoria/75	$10^{7.0}$	i.t.	10	7[d]	1 (1–3)	5.7 (2–10)	6/6	208	100	0	2	8
		i.n.	4	4[d]	1 (1)	6 (5–7)	3/3	32	100	0	4	0
A/New Jersey/76	$10^{6.0}$	i.t.	10	1[d]	2	3	6/6	40	100	2	6	2
		i.n.	4	3	1 (1–2)	4 (2–6)	3/3	12	100	2	2	0
Allantoic fluid		i.t. + i.n.	8	0			0/6	0	0	8	0	0

[a] Excluding those animals autopsied on day 4 to 6.

[b] RTI is defined as an illness that was present on a minimum of at least 2 consecutive days during which at least two of the following signs were observed: rhinorrhea, sneezing, excessive lacrimation, or conjunctivitis.

[c] Systemic illness consisted of an RTI plus the presence of dyspnea, lethargy, depressed response to stimulation, or marked anorexia.

[d] Significant difference at $P < 0.05$ level, Fisher Exact Test.

(6 days) occurred in a manner identical to that of animals inoculated i.t. Although the magnitude of the serological response was significantly less than that of the i.t.-inoculated group, each of the animals inoculated i.n. with the Victoria strain developed a fourfold or greater increase in serum HAI titer. Each monkey developed clinically recognizable RTI characterized rhinorrhea and conjunctivitis. Illness was first detected on day 2, was maximal on days 3 and 4, and subsided by day 9. None of the animals became lethargic, dyspneic, anorectic, or depressed.

Of the four animals inoculated i.n. with A/New Jersey/76 virus, two developed an upper RTI and two remained healthy. Three of the four animals shed virus, and each developed a fourfold or greater rise in serum HAI antibody.

Radiographic examination. The chest X rays were read under code by John Doppman, Chief of Diagnostic Radiology, National Institutes of Health. Seven of the eight animals that developed signs of systemic illness after i.t. infection with the Victoria/75 virus showed radiographic evidence of pulmonary disease, as did one of the two animals that failed to manifest systemic illness. Typical findings consisted of bilateral lower and middle lobe infiltration more obvious on the lateral than AP projection. None of the remaining 26 animals inoculated with A/Victoria/75 (i.n.), A/New Jersey/76 (i.t. or i.n.), or sterile allantoic fluid (i.n. and i.t.) developed significant radiological changes.

Hematological evaluation. Leukocyte levels were found to vary widely with the majority of the individual animals exhibiting marked fluctuation from day to day before inoculation. After infection there was no consistent pattern of leukopenia, leukocytosis, or relative lymphocytosis.

Pathology. Four of the 10 animals receiving A/Victoria/75 virus (i.t.) were sacrificed and necropsied on day 4 to 6 post-inoculation. Gross inspection of the respiratory tract revealed focal areas of hyperemia in the trachea; each of the lobes of the lung contained patchy, red areas of consolidation measuring on the average 4 to 5 cm in diameter. Histological examination disclosed mild submucosal inflammation of the nasal turbinates. Erosion of the tracheal and bronchial epithelium was accompanied by loss of cilia, squamous metaplasia, focal areas of hemorrhage, and submucosal infiltrations of mononuclear cells. Microscopically, the alveolar walls were thickened by infiltration of mononuclear cells and deposits of proteinaceous material within the terminal air spaces.

Of the 10 animals receiving A/New Jersey/76 virus i.t., 4 were sacrificed. Gross lesions were not found in any of these animals. Histological examination, however, disclosed changes in the trachea and lungs that were much less severe, but otherwise similar to those found in the animals inoculated i.t. with the A/Victoria/75 strain. With the exception of submucosal inflammation of the nasal turbinates, examination of animals inoculated i.n. with either the A/Victoria/75 or A/New Jersey/76 strain revealed no pathological changes. Necropsy of animals inoculated i.n. and i.t. with normal allantoic fluid disclosed no abnormalities.

DISCUSSION

The medical and epidemiological literature is replete with reports suggesting that nonhuman primates may be infected either naturally or artificially with strains of influenza virus pathogenic for humans (1–9, 11, 13–16). There is little evidence, however, that such infection in a healthy simian host leads directly to the occurrence of easily recognizable lower respiratory tract disease. To produce signs of illness, lung lesions and a significant serological response, it has been necessary to administer virus either by the i.t. route or by prolonged exposure in an aerosol chamber; i.n. administration has usually produced an asymptomatic infection and a relatively weak immune response. The severity of illness and magnitude of the antibody response are dependent upon the infecting strain of influenza virus, the dose of the inoculum, the route of inoculation, and the species of primate employed. Environmental alteration (i.e., nutritional deprivation and exposure to cold) has also been shown to influence the course of illness (16).

Although lower respiratory tract disease has been induced in primates by direct exposure to influenza virus, it has not been predictable and verification has usually required autopsy of the experimental host. In the present study, 8 of the 10 animals inoculated i.t. with 10^7 $TCID_{50}$ of influenza A/Victoria/75 virus developed clinically recognizable systemic illness; 7 of these 8 animals had pulmonary infiltration that was detectable radiologically. One monkey that was inoculated i.t. and whose signs were localized to the upper respiratory tract also had pulmonary X-ray changes. i.n. inoculation of the same volume and dose of A/Victoria/75 virus produced only mild upper respiratory symptoms in each of four monkeys tested; there were no pulmonary changes detected radiologically. Two of the 10 monkeys inoculated i.t. with 10^6 $TCID_{50}$ of A/New Jersey/76 virus had a brief systemic illness, 6 had upper respiratory symptoms, and 2 remained well; none had significant changes on chest X ray. Consistent with the observation of more severe illness in monkeys receiving A/Victoria/75 was the higher frequency of virus

shedding (7 of 10 animals), as compared to those monkeys inoculated with A/New Jersey/76 (1 of 10 animals). The factors underlying the difference in virulence of A/Victoria/75 and A/New Jersey/76 viruses cannot be deduced on the basis of our findings because of the difference in passage history of the two viruses and the 10-fold difference in virus dose administered, although each was 100% infective.

The results of the present study do indicate, however, that the cebus monkey is susceptible to respiratory tract infection with influenza A virus. Most important, however, are the observations that systemic illness and lower respiratory tract disease were reproducible and that systemic illness correlated with pathological pulmonary changes which could be confirmed radiologically. It is therefore proposed that this nonhuman primate species may provide a model for influenza A viral infections in which illness and lower respiratory tract disease may be followed objectively without sacrifice of the animal.

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