Aerosol Exposure of Monkeys to Influenza Virus.* (30315)

SAMUEL SASLAW AND HAROLD N. CARLISLE
Division of Infectious Diseases, Department of Medicine, Ohio State University College of Medicine, Columbus

Previous studies from this laboratory(1) showed that intratracheal inoculation of rhesus monkeys with the PR8 strain of influenza virus resulted in mild illness in 2 of 4 monkeys; after intranasal inoculation, no clinical signs of illness were observed. Results of studies by others(2-5) have varied with the influenza virus strain and monkey species employed. In all, virus was administered intratracheally or intranasally, and in general, clinical evidence of disease was minimal or absent.

The development of quantitative procedures for exposure of experimental animals to aerosols of infective organisms(6) suggested reinvestigation of the response of monkeys following challenge with influenza virus by these methods. The present report describes the clinical, hematologic and serologic response of rhesus and cynomolgus monkeys after aerosol exposure to the PR8 strain of influenza virus.

Materials and methods. Challenge of young adult monkeys, *Macaca mulatta* and *M. irus*, was conducted in a Model 3 Henderson apparatus(6) by previously described methods (7,8). The aerosol generator was charged with mouse lung filtrate containing about 1.0×10^8 mouse intranasal LD50 per ml of the PR8 strain of influenza A virus. Monkey doses were calculated from studies of concurrently exposed 14-16 g white mice. Intratracheal inoculation of allantoic fluids(9) containing strains A/PR8/34, Swine/1976/31, A2/Japan/305/57, and B/Great Lakes/1739/54 was performed as described previously(1).

Physical examinations, hematologic and serologic studies were carried out for 2 weeks prior to challenge. After challenge, monkeys were examined twice daily. Femoral vein blood was used for blood counts and C-reactive protein (CRP) tests daily or every other day, and weekly for serologic studies. Blood samples and material collected from the nasopharynx with a cotton-tipped flexible wire were frozen immediately, and stored at —65°C pending attempts to isolate virus by amniotic inoculation of eggs(9). Tests for presence of CRP were performed as recommended by Schieffelin and Co., New York, using their commercial anti-human CRP serum. Serologic response was followed by hemagglutination-inhibition (H-I) test(9,10).

Results. Aerosol challenge of rhesus monkeys. Twelve monkeys (Table I) exposed 2-4 minutes to aerosols containing 1.6×10^4 to 2.4×10^5 mouse aerosol LD50 per liter retained about 1.6×10^5, 3.2×10^4, 9.8×10^4 and 3.6×10^5 LD50 in 4 experiments, respectively, based on a respiratory volume of about 1 liter per minute(11) and on 50% retention of particles(12). Five of 12 exhibited clinical signs of illness after exposure (Table I). Two (Nos. 1, 10) became moderately ill and 3 (Nos. 6, 7, 9) showed minimal transient symptoms. Monkeys 1 and 10 reacted similarly; both became weak, anorectic and listless on the 2nd post-exposure day (PED). Monkey 10 remained so during the 3rd PED, reacted sluggishly to handling, and sat quietly in the cage with head hanging and arms clasped to the body. On stimulation, it moved unsteadily about the cage. Improvement was noted during the 4th and 5th PED, and the animal appeared normal by the 6th PED. Monkey 1 showed chills and fever (103.6°F) on the 2nd PED in addition to symptoms through the 4th PED similar to those seen in monkey 10, but recovered more slowly, and was not active and well until the 8th PED. Temperatures remained normal in both monkeys except for the one day in monkey 1. CRP was 1+ and 7+ on the 2nd and 3rd days, respectively, in monkey 10, but negative thereafter.

Monkeys 6, 7 and 9 were listless and anorectic on the 3rd to 5th PED, 5th PED only, and 3rd and 4th PED, respectively. On

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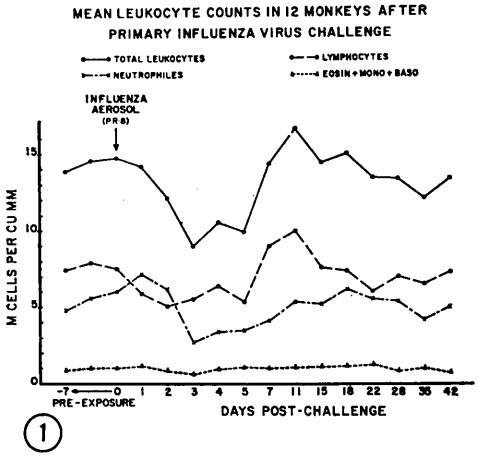
TABLE I. Response of Rhesus Monkeys After Aerosol Challenge with Influenza Virus, Strain PR8.

Exp No.	I					II				III		IV
Mouse aerosol LD50	1.6 × 10⁵					3.2 × 10⁴				9.8 × 10⁴		3.6 × 10⁵
Monkey No.	1	2	3	4	5	6	7	8	9	10	11	12
Symptoms	Moderate	—	—	—	—	Mild	Mild	—	Mild	Moderate	—	—
Fever	+	—	—	—	—	—	—	—	+	—	—	—
C-reaction protein	—	—	—	—	—	—	—	—	—	+	—	+
Neutropenia	+	+	+	+	Minimal	+	+	+	Minimal	+	+	+
Lymphopenia	—	+	+	+	"	+	+	—	"	—	—	—
H-I titer — base line	—	—	—	20	—	—	—	—	—	—	—	—
" — 1 week	20	640	640	1280	1280	20	10	10	40	10	20	10
" — 2 "	320	2560	2560	2560	1280	160	20	160	160	80	320	20
" — 3 "	320	2560	2560	2560	1280	640	20	320	320	640	1280	40
" — 4 "	640	1280	1280	1280	640	640	40	320	320	320	1280	80

stimulation, however, all appeared normal. The rectal temperature was 104.0°F in monkey 9 on the 3rd PED. The highest pre-exposure temperature had been 103.1°F. All tests for CRP were negative in monkeys 6, 7 and 9.

Definite hematologic changes were observed in all 12 monkeys (Table I). Ten of 12 showed significant neutropenia which was maximal on the 3rd through 7th PED (Fig. 1). Five of 10 also showed significant lymphopenia which was most evident on the 1st through 5th PED. Two (Nos. 5, 9) of 12 monkeys in which neutropenia was not obvious showed minimal depression in both neutrophiles and lymphocytes so that total leukocyte counts were lower than base-line. A



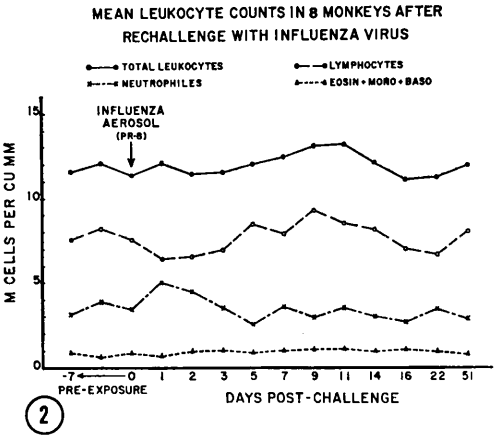


FIG. 1. Mean leukocyte counts in 12 monkeys after primary influenza virus challenge.

FIG. 2. Mean leukocyte counts in 8 monkeys after rechallenge with influenza virus.

TABLE II. Response of Cynomolgus Monkeys After Aerosol Challenge with Influenza Virus, Strain PR8.

	Exp V			
Mouse aerosol LD50	6.3 × 10^5			
Monkey No.	13	14	15	16
Symptoms	Mild	Mild	—	Moderate
Fever	+	—	—	—
C-reactive protein	+	—	—	+
Neutropenia	+	+	—	+
Lymphopenia	+	+	—	+
H-I titer — base line	—	—	—	—
" — 1 week	20	20	80	20
" — 2 "	640	80	80	320
" — 4 "	1280	80	80	640

slight, initial *increase* in neutrophiles on the 1st and 2nd PED masked the effect of early lymphopenia so that total leukocyte counts were not markedly depressed until the 3rd PED (Fig. 1). Reciprocal lymphocytosis was noted in 4 of 10 monkeys on the 7th and 11th PED. No significant changes in hemoglobin or hematocrit value were noted.

Antibody response was followed by the H-I test using strains A/PR8/34, A2/Japan/305/57, Swine/1976/31, A'/FM1/47, A'/PR301/54, A'/Ann Arbor/1/57, A'/Denver/1/57, B/Lee/40, and B/Great Lakes/1739/54 as antigens. The response in all 12 monkeys was directed only against the challenge strain, PR8 (Table I); peak titers from 1:40 to 1:2560 were observed 1-4 weeks post-challenge.

Blood samples and nasopharyngeal material taken daily for 5 days after exposure were inoculated amniotically into eggs in an attempt to isolate influenza virus. Virus was not recovered from any of the samples.

Aerosol challenge of cynomolgus monkeys. The response of 4 cynomolgus monkeys after aerosol challenge was similar to that observed in rhesus monkeys (Table II). Monkey 16 exhibited moderate illness grossly similar to that observed in rhesus monkeys 1 and 10 above whereas cynomolgus monkeys 13 and 14 showed minimal symptoms as seen in rhesus monkeys 6, 7 and 9 (Table I). Two animals, 13 and 16, exhibited positive CRP tests from the 2nd to 8th days and 2nd to 18th days, respectively. Monkey 13 also showed a minimal elevation in the rectal temperature on the 2nd and 3rd PED. All 3 cynomolgus monkeys that became ill exhibited neutropenia and lymphopenia whereas counts were normal in the animal that showed no symptoms. All 4 cynomolgus monkeys showed significant rises in H-I titer against the challenge strain, PR8, only. Peak titers of 1:80 to 1:1280 were noted 2 or 4 weeks after exposure (Table II).

Intratracheal challenge. For comparative purposes, 2, 2, 1 and 1 rhesus monkeys each were inoculated intratracheally, under light ether anesthesia, with 2.0 ml of allantoic fluid containing PR8, Swine, Great Lakes or Asian strains, respectively (Table III). Three controls each received 2.0 ml of normal allantoic fluid while 1 control was anesthetized, and a dry needle was introduced into the trachea. The allantoic fluids containing PR8, Swine, Great Lakes and Asian strains showed chicken cell hemagglutination titers of 1:1280, 1:640, 1:160 and 1:640, respectively. Intranasal titrations of the PR8, Swine and Asian allantoic fluids in mice showed that monkeys received approximately 2.0 × 10^6, 8.0 × 10^5 and 4.0 × 10^2 mouse intranasal LD50, respectively. The Great Lakes allantoic fluid was not lethal for mice.

One of 2 monkeys (No. 17, Table III) which received the PR8 strain, became listless and anorectic on the 2nd PED. During the 3rd and 4th PED, it sat quietly in the cage with head hanging and hands clasped over the top of the head. Occasional chills were observed. When stimulated, the monkey would move unsteadily in the cage. Recovery was rapid, and the animal appeared normal by the 6th day. Temperatures were normal during this period and subsequently. One of 2 monkeys (No. 19), which received the Swine strain, reacted similarly from the 2nd through 4th PED except that chills were not observed. Infrequent coughing was noted on the 3rd day only. Monkey 20, which also received the Swine strain, was listless and anorectic on the 3rd day only. No outward evidence of disease was noted in monkeys receiving Great Lakes or Asian strains or in the controls.

The hematologic response was very much like that observed after aerosol challenge. Neutropenia and lymphopenia were observed

TABLE III. Response of Rhesus Monkeys After Intratracheal Challenge with Influenza Virus Allantoic Fluids.

	Exp VI						
Challenge strain	PR8		Swine		Great Lakes	Asian	
CCA titer of allantoic fluid	1:1280		1:640		1:160	1:640	
Mouse I.N. LD50 (2.0 ml)	2.0×10^6		8.0×10^5		—*	4.0×10^2	Controls†
Monkey No.	17	18	19	20	21	22	23–26
Symptoms	Moderate	—	Moderate	Mild	—	—	—
Fever	—	—	—	—	—	—	—
C-reactive protein	—	—	—	—	—	—	—
Neutropenia	+	+	—	+	+	+	—
Lymphopenia	—	+	+	+	—	+	—
H-I titer‡— base line	—	—	—	—	—	—	—
" — 5 days	80	20	—	—	—	—	—
" —10 "	1280	320	20	40	—	20	—
" —15 "	640	320	80	80	10	80	—
" —20 "	640	320	80	80	20	160	—
" —30 "	640	320	80	80	20	160	—
" — 6 weeks	320	320	80	80	20	80	—
" —15 "	320	160	80	40	20	160	—
" —20 "	320	160	80	80	10	160	—

* Not lethal for mice inoculated intranasally.
† 23, 24, 25 received normal allantoic fluid, 26, dry needle inserted.
‡ H-I titer to challenge strain.

in 5 of 6 and 4 of 6, respectively, from the 2nd through 5th and 1st through 4th PED, respectively (Table III). No change in blood picture was observed in 4 control animals.

All 6 monkeys inoculated with viable virus showed significant increases in H-I titer (Table III). In each case, antibodies were directed against the challenge strain only. Monkeys No. 17 and 18, 19 and 20, 21, and 22 showed peak titers of 1:1280 and 1:320, 1:80 and 1:80, 1:20, and 1:160, respectively, against the PR8, Swine, Great Lakes and Asian strains, respectively.

Attempts to isolate influenza virus from blood samples and nasopharyngeal material taken daily for 5 days after inoculation were unsuccessful.

Aerosol rechallenge experiments. The 6 monkeys (Nos. 17-22) inoculated intratracheally with infectious allantoic fluids *(vide supra)* were rechallenged 5 months later by exposure to an aerosol of the PR8 strain. Each received about 1.6×10^5 mouse LD50. One normal monkey was similarly challenged.

None of the monkeys inoculated previously with allantoic fluids showed signs of illness after aerosol rechallenge; temperatures remained normal and all tests for CRP were negative. Control monkey No. 1 became moderately ill and is described in detail in Table I (response after primary aerosol challenge) as are other controls in this phase.

In a second experiment, 2 monkeys (Nos. 2, 3) which had been challenged by PR8 aerosol (Table I) were rechallenged 6 months later, together with 2 controls (Nos. 10, 11) by exposure to an aerosol of the PR8 strain. Each retained about 9.8×10^4 mouse aerosol LD50. Neither of the previously exposed animals showed any signs of illness after rechallenge, whereas 1 of 2 controls became moderately ill (Table I).

Thus, in 2 rechallenge experiments, none of 8 animals previously challenged with influenza virus intratracheally or by aerosol showed signs of illness, whereas 2 of 3 normal monkeys became moderately ill. The 4 which had received PR8 previously (Nos. 2, 3, 17, 18) showed H-I titers of 1:160, 1:320, 1:320 and 1:160, respectively, against the PR8 strain at the time of rechallenge and 3 of 4 showed significant 4-fold titer rise after rechallenge. Two monkeys (Nos. 19, 20) and 1 (No. 22) previously inoculated intratracheally with Swine and Asian strains, respectively (Table III) showed no anti-PR8 H-I antibody at the time of rechallenge but showed titers of 1:80, 1:80 and 1:160 against their original challenge strain. PR8 titers showed significant rise in all 3 and heterolo-

TABLE IV. Serologic Response of Rhesus Monkeys After Aerosol Rechallenge with Influenza Virus, Strain PR8.

	Monkey No.	Original challenge strain	H-I test antigen strain	Base line*	H-I titer—Weeks post rechallenge 1	2	3	4	7
	17	PR8	PR8	320	320	640	640	1280	1280
	18	"	"	160	640	640	640	320	320
	19	Swine	Swine	80	320	320	160	160	320
			PR8	—	20	80	160	80	40
Exp VII—1.6 × 10^5 mouse aerosol LD50	20	"	Swine	80	160	320	320	320	320
			PR8	—	40	80	80	80	40
	21	Great Lakes	GL	10	10	10	20	10	20
			PR8	—	80	80	160	160	160
	22	Asian	Asian	160	160	320	320	320	160
			PR8	—	—	40	160	320	320
	1	None	"	—	20	320	320	640	640
	2	PR8	"	160	320	640	320	ND†	ND
Exp VIII—9.8 × 10^4 mouse aerosol LD50	3	"	"	320	320	320	320	ND	ND
	10	None	"	—	10	80	640	320	320
	11	"	"	—	20	320	1280	1280	640

* 5-6 months post-original challenge. † Not done.

gous titers rose significantly in both monkeys which had received Swine virus, but not in the monkey which had received Asian. The monkey (No. 21) which had received the antigenically distinct Great Lakes B strain intratracheally also showed no anti-PR8 antibody and had a 1:10 titer against the Great Lakes strain at the time of rechallenge. After rechallenge, this animal showed significant increase in anti-PR8 titer only.

The white cell response after aerosol rechallenge of 8 monkeys previously inoculated intratracheally or exposed to an aerosol was not depressed (Fig. 2) as compared to that observed after primary challenge (Fig. 1).

Discussion. The few studies(1-5) on experimental influenza infection in monkeys have yielded divergent opinions as to the usefulness of this animal in influenza research. Numbers of animals have been small, and inoculation has been intranasal or intratracheal. The present study showed that aerosol challenge with the PR8 strain of influenza virus resulted in non-fatal illness in 5 of 12 and 3 of 4 rhesus and cynomolgus monkeys, respectively; leukopenia was observed in 13 of 16, and all exhibited significant rise in H-I titer against the homologous strain, only. Intratracheal inoculation resulted in illness in 1 of 2 and 2 of 2 monkeys receiving PR8 and Swine strains, respectively, but not in single monkeys receiving Asian or Great Lakes strains; all showed leukopenia and specific antibody responses, however. The results with the PR8 strain are in agreement with previous studies(1) from this laboratory. Similarly, aerosol exposure resulted in observable illness in about half the monkeys challenged in contrast to no illness following intranasal inoculation(1) although similar hematologic and serologic responses were observed.

Aerosol rechallenge of 4 monkeys suggested that contact 5-6 months previously with PR8, intratracheally, or by aerosol, altered the response; none showed illness or leukopenia. Similar results were observed in 2 monkeys previously exposed to Swine and 1 each exposed to Asian and Great Lakes B strain. It is difficult to assess the response in the latter monkeys which had received heterologous strains, and further studies are in progress to determine whether these strains could alter the response to PR8 challenge.

Intratracheal or intranasal inoculation in anesthetized monkeys represents an artificial mode of infection. Aerosol challenge with a Henderson apparatus affords a more natural means of exposure not requiring trauma or anesthesia. Combining the clinical, hematologic and serologic findings, one has sign-posts of disease more closely simulating that observed in man. These studies suggest that

the monkey may be a useful experimental animal in the broad study of influenza, particularly in the present day development of potential antiviral agents.

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Human Aorta Cells for Isolation and Propagation of Rhinoviruses.* (30316)

C. ALAN PHILLIPS, JOSEPH L. MELNICK AND CATHERINE A. GRIM

WHO International Reference Centre for Enteroviruses, Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

A cell line derived from human aorta containing atheromatous plaques (A-39) has recently been shown to support the growth of a large number of viruses(1). During the past 2 years, we have used these cells extensively in the isolation and propagation of rhinoviruses(2) and they have proved a useful addition to WI-38(3) and human embryonic kidney cells(4).

Materials and methods. All materials and methods employed in this study have been described(5).

Results. While studying upper respiratory tract infections in a population of college students, 19 rhinoviruses were isolated from 111 specimens between October 8, 1963 and April 30, 1964 (period 1), 51 rhinoviruses were isolated from 107 specimens between September 29, 1964 and December 31, 1964 (period 2), and 22 rhinoviruses were isolated from 55 specimens between January 1, 1965 and March 6, 1965 (period 3). The isolates were shown to be RNA viruses which were acid-labile at pH 3.0 and ether-stable, thus possessing the properties of a rhinovirus(4). Those that were viewed by electron microscopy measured 18-23 mμ.

During period 1 (Table I) 18 of the 19 rhinoviruses were isolated in A-39, while 11 of 19 were isolated in WI-38, and only 1 was detected in HEK. Seven of these strains were isolated in A-39 alone but later grew well in WI-38, while 1 strain was isolated only in WI-38 but then grew well in A-39.

During period 2, 17 of 51 rhinovirus strains were isolated in A-39, 24 of 51 were isolated in WI-38, and 43 of 51 were detected in HEK. Of these, none were isolated in aorta alone, while 4 were isolated in WI-38 alone, and 23 were isolated only in HEK.

During period 3, 17 of 22 rhinovirus strains were isolated in A-39 and WI-38, while only 9 were isolated in HEK. Three of these strains were isolated in A-39 alone, 4 were isolated in WI-38 alone, and one was detected only in HEK. Isolations in more than one cell line were common during the 3 study periods as can be seen in Table I. It

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