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Attempts to Infect Rhesus Monkeys with Human Type 4 Adenovirus (33347)

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Adenovirus infections constitute a major cause of acute respiratory disease in military recruits (1–3). For several years respiratory disease in this group was controlled by parenteral administration of polyvalent formalin-inactivated vaccines which incorporated adenovirus types 3, 4, and 7 (4,5). Increasing concern with potential oncogenicity of some adenoviruses and with the oncogenic properties of the contaminating SV40 virus led to the suspension of routine use of these vaccines (6–8).

In 1966 Chanock and associates (6) reported the development of a live adenovirus type 4 vaccine for oral administration as enteric-coated tablets. The unattenuated virus used in this vaccine was grown in the WI-38 human diploid fibroblast cell culture and has been shown to be free of known adventitious agents and oncogenic activity in the usual test systems. Volunteer studies have shown that virus administered in this manner bypasses the upper respiratory tract, selectively infects the lower intestine, and is readily excreted in the stools of vaccinees (9). Virus spread to nonimmune contacts did not occur under the usual conditions of life in the barracks (9).

Later studies suggested that in some families the vaccine virus spread from immunized to nonimmunized spouses (10). It seemed worthwhile to explore the use of the simian primate as a laboratory model in studying adenovirus infections. A review of the literature disclosed that others had failed to infect monkeys with human adenoviruses (11). This report describes our attempts to infect rhesus monkeys with human type 4 adenovirus and to determine communicability among uninoculated cagemates.

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Materials and Methods. Virus. Adenovirus type 4, prototype strain RI67, was obtained from Dr. Kendall O. Smith. The passage history from isolation was as follows: Human trachea cell culture/4, HeLa/17, KB/8+. Infectivity titer of our stock preparation as determined by cytopathic effect (CPE) in primary human embryonic kidney (HEK) cell cultures was $10^{8.5}$ $TCID_{50}$/0.1 ml of inoculum after 7-days incubation in stationary racks at 37°.

Animals. Eighteen juvenile (4–6 pound) rhesus (*Macaca mulatta*) monkeys were divided into three equal groups and housed in pairs. One animal from each pair served as an uninoculated cagemate control. Monkeys from groups 1 and 2 were inoculated with 1.0 ml ($10^{9.5}$ $TCID_{50}$) stock adenovirus intramuscularly (i.m.) and intravenously (i.v.), respectively. Animals in group 3 received 0.25 ml of virus intranasally (i.n.) while under deep barbiturate anesthesia.

Cell cultures. Tube cultures of primary human embryonic kidney (HEK) cells were obtained from Microbiological Associates, Inc., Bethesda, Maryland and were used for virus isolation and antibody assays. The HEK cells were maintained with medium 199 containing 2% fetal calf serum and antibiotics (100 units of penicillin G, 100 μg of streptomycin sulfate, and 125 units of nystatin (Mycostatin)/ml).

Collection and processing specimens. Blood samples, throat and anal swabs were collected from all animals prior to and on the day of inoculation. Subsequent specimens were collected on 2, 5, 7, 9, 12, and 14 days after inoculation from every animal, and, in addition, on days 19 and 21 from the uninoculated cagemate controls. Two additional blood specimens were obtained from the cagemate controls 28 and 35 days after inoculation. Blood for virus isolation was collected using heparinized syringes and tubes. After remaining at 4° for 12–16 hr the buffy coat layer was removed and immediately inoculated into cell cultures. Throat and anal swabs were immersed in 2 ml of chilled phosphate buffered saline (12) containing 0.5% bovine plasma albumin, streptomycin (100 μg/ml) and penicillin G (100 units/ml). Fluids from the anal swabs were centrifuged at 3000 rpm for 30 min at 4° and the supernatants were inoculated into cell cultures. When the specimens could not be inoculated immediately after collection they were stored at —70° until tested. Sera for serological testing were separated from freshly coagulated venous blood by centrifugation at 2000 rpm for 15 min and stored at —20° until used.

Virus isolation. One-tenth ml of each specimen was inoculated into each of four tube cultures of HEK cells from which the medium was previously removed. Following adsorption at 37° for 1 hr, 1 ml of fresh maintenance medium was added. Tubes were incubated at 37° in stationary racks. The fluid medium was changed 24 hr after inoculation and every 5–6 days thereafter. Cultures were observed for CPE every 2–3 days and were held for 28 days. Routine passage was not performed unless toxic nonspecific degeneration occurred, in which case cells and fluid were passaged into fresh cell cultures which were then observed for 28 days.

Isolates were identified by neutralization tests performed by mixing equal quantities of heated (56° for 30 min) specific adenovirus type 4 immune rabbit serum diluted 1:5 and decimal dilutions of the virus isolate; a control nonimmune rabbit serum treated similarly was tested with each isolate and a known type 4 adenovirus was included in each test as the positive virus control; after incubation at room temperature (20–25°) for 1 hr, 0.2 ml of the virus–serum mixture was inoculated into each of 2 tube cultures of HEK cells; following adsorption for 1 hr at 37°, 1 ml of maintenance medium was added. The tubes were incubated and end points were calculated at 7 days by the Kärber method (13). Under such conditions the specific adenovirus type 4 immune rabbit serum had a LNI[2] of 3.5–4.0. An isolate was considered adenovirus type 4 if it could be neutralized within this same range.

[2] LNI = log neutralization index, which represents the difference in virus titer between the series containing the control nonimmune rabbit serum and the series containing the specific adenovirus type 4 antiserum.

TABLE I. Adenoviruses Recovered from Inoculated Monkeys and Cagemate Controls.

Monkey no. (route of inoc.)[a]	Isolate no.	Source	Time of isolation (days p.i.)[b]	Onset of CPE[d] (days p.i.)	Identified as adenovirus type 4[c]	4-fold AB[e] titer rise	
						Neut	CF
R1706 (i.m.)	1	Stool	2	15	No[f]		
	2	Stool	5	14	No		
	3	Stool	7	12	No	Yes	Yes
	4	Stool	9	12	No		
R1710 (i.v.)	5	Buffy coat	2	10	Yes		
	6	Stool	5	14	Yes	Yes	Yes
R1711 (CC)	7	Stool	14	20	No[f]		
	8	Stool	21	20	No	No	No
R1714 (i.v.)	9	Stool	2	12	Yes	Yes	Yes
R1715 (CC)	10	Stool	21	15	No[g]	No	No
R1718 (i.n.)	11	Stool	2	12	Yes	Yes	Yes
R1720 (i.n.)	12	Stool	2	12	Yes	No	No

[a] Route of inoculation: i.m. = intramuscular; i.v. = intravenous; CC = cagemate control; and i.n. = intranasal.

[b] Days postinoculation.

[c] By neutralization test.

[d] Cytopathic effect.

[e] Antibody.

[f] LNI (log neutralization index) = 0.

[g] LNI = 1.0.

Antibody assays. Serum neutralizing antibody was determined by mixing a constant virus dose (56–100 $TCID_{50}$) of the stock preparation of type 4 adenovirus with serial 2-fold dilutions of heated sera (13). All sera collected during a single day were assayed in the same test. Complement-fixing (CF) antibody was determined by a procedure used in our laboratory (14).

Results and Discussion. No viruses were isolated from specimens obtained before inoculation. From 162 blood, anal, and throat specimens collected from the inoculated monkeys over a 14-day period after inoculation, 9 viruses with typical adenovirus CPE were obtained. The positive specimens were obtained from 5 of the 9 inoculated monkeys (Table I): 8 of the isolates came from anal swabs and 1 from buffy coat. No isolations were made from throat specimens. From 216 blood, anal and throat specimens collected from the 9 cagemate controls over a 21-day period, 3 isolates with typical adenovirus CPE were recovered from anal swab specimens obtained from 2 animals.

Five isolates (Table I, nos. 5, 6, 9, 11, and 12) were identified as adenovirus type 4 by neutralization. All were obtained from inoculated monkeys; 3 were recovered from 2 of the i.v. inoculated monkeys, and 2 from the i.n. inoculated animals. Four of 5 isolates were from anal swabs, the fifth from buffy coat. Although all isolates were identified as adenoviruses by CF tests, no attempt was made to type those which were not type 4 adenovirus. No other viruses were isolated in our HEK cell culture system.

Neutralizing antibody was not detected in any of the sera obtained before inoculation. However, there was a low CF antibody titer in a few of these same sera; no virus isolations were made from the animals with preexisting CF antibody and rises in antibody titer were not observed. Three of 4 monkeys, from which type 4 adenoviruses were recovered, developed neutralizing antibody 2

TABLE II. Neutralization and Complement-Fixation Tests with Sera from Monkeys from which Type 4 Adenovirus Was Isolated.

Monkey no. (route of inoc.)[a]	Days post-inoculation	Antibody titer[c]	
		Complement fixation	Neutralization
R1710 (i.v.)	(−7)[b]	<4	<4
	0	<4	<4
	7	8	<4
	14	8	8
R1714 (i.v.)	(−7)	<4	<4
	0	<4	<4
	7	4	128
	14	4	256
R1718 (i.n.)	(−7)	<4	<4
	0	<4	<4
	7	8	<4
	14	16	8
R1720 (i.n.)	(−7)	<4	<4
	0	<4	<4
	7	<4	<4
	14	<4	<4

[a] Route of inoculation: i.m. = intramuscular; i.v. = intravenous; and i.n. = intranasal.
[b] Serum obtained 7 days prior to inoculation.
[c] Reciprocal of serum dilution.

weeks following virus administration (Table II). All 3 animals also developed CF antibody during the same period. Neutralizing antibody was not detected in the sera of the cagemate controls from which non-type 4 adenoviruses were obtained.

There is evidence to suggest that adenovirus infection and replication did occur in several inoculated animals. Isolates nos. 6, 9, and 11 (Table I) were recovered from anal swabs 2–5 days after inoculation. The 3 animals (R1710, R1714, and R1718) from which these viruses were recovered developed a 4-fold or greater rise in neutralizing and CF antibody titers over a 2-week period. The single isolate from buffy coat was obtained from a monkey (R1710) inoculated intravenously. The fact that this isolate was recovered 2 days after inoculation suggests that this animal had a viremia which persisted. An additional isolate from the stool of this same animal and a significant rise in neutralizing antibody titer are evidence that virus replication occurred. Monkey R1714 developed the highest level of neutralizing antibody (1:256) and a slight rise in CF antibodies (Table II). The isolate recovered from this animal was detected in the stool as early as 2 days following intravenous inoculation of virus (Table I). The serological findings suggest that this animal was infected.

On the other hand, the results can be interpreted in another way. Kosunen and Kääriänen (15) reported that parenteral inoculation of nonpathogenic poliovirus or phage ΦX174 into guinea pigs resulted in the gradual elimination of the viruses in urine, bile, and stool over a 2–4-day period and a resorptive, steadily declining viremia for 2–3 days. It could be postulated that the sporadic virus isolations in monkeys R1710, R1714, and R1718 represent passive excretion in the absence of replication. The antibody responses might be a reflection of the large virus dose administered. Our studies do not resolve this question.

The unusually high neutralizing antibody titer seen in monkey R1714 is difficult to explain. When Hilleman and his associates (16) administered live adenovirus to man parenterally, the usual neutralizing antibody response approximately 2 weeks after inoculation was of the order of magnitude of 1:16. This response in the more susceptible human hosts is considerably lower than that seen in the monkey. However, the high neutralizing antibody titer seen in the same animal was not necessarily incompatible with its low level CF antibody response. For instance, when navy recruits, free of neutralizing antibody, were infected with type 4 virus by the enteric route only 50% developed CF antibody, while 100% developed neutralizing antibody (9). Apparently, even in humans, the CF antibody response may be quite variable.

Isolates nos. 11 and 12 (Table I) were recovered from anal swabs collected 2 days after intranasal inoculation of 2 different monkeys (R1718 and R1720). These isolates were identified as type 4 adenovirus. Although monkey R1718 developed significant rises in neutralizing and CF antibody titers, monkey R1720 failed to develop antibody.

This suggests that the former animal was infected, whereas the isolate obtained from the latter may represent merely stool excretion after passage through the intestinal tract. The possibility exists that both cases represented a gradual elimination of virus in the stool in the absence of infection and replication.

Monkey R1706 (Table I) shed virus rectally for at least a week. Although repeated neutralization tests failed to identify these isolates (nos. 1–4) as type 4 adenovirus, this animal developed a 64-fold rise in neutralizing antibody titer and a 32-fold rise in CF antibody titer 1 week after inoculation. Antibodies were not detected before inoculation. One possible explanation for our failure to identify these isolates as type 4 is that this animal was infected with 2 adenoviruses and we were able to recover only one of them.

The 3 isolates recovered from the cagemate controls (R1711 and R1715) of monkeys R1710 and R1714 could not be identified as type 4 adenovirus by neutralization tests but did fix complement in the presence of type 4 antibody. These isolates could have been simian adenovirus types, but we did not perform tests to resolve this. In any case, it appears that communicability between inoculated and uninoculated cagemates could not be demonstrated.

The lack of pathogenicity for commonly employed laboratory animals is considered a general characteristic of human adenoviruses. At present, information on pathogenesis and immunogenicity must be derived from volunteer studies. The desirability of developing an animal model for human adenovirus studies seems obvious.

Earlier attempts to infect rhesus monkeys with human adenovirus by the intracranial, intranasal, and subcutaneous routes have been unsuccessful. Our studies indicate that although it may be possible to infect rhesus monkeys with human adenoviruses, they are not readily susceptible to infection and would not serve as useful models for vaccine testing. Despite using a large virus dose, infection was not uniformly accomplished. Communicability between inoculated and uninoculated cagemates could not be demonstrated despite the obvious opportunities for anal–oral spread in confined housing and under intimate and unsanitary conditions. Additional experience with human adenoviruses in rhesus monkeys is necessary before conclusive proof of infection in this animal is established.

Summary. In searching for an animal model for human adenovirus studies we have attempted to infect rhesus monkeys with a human type 4 adenovirus. The results were inconclusive but we have some evidence to suggest that infection and replication might have occurred in several animals. Communicability among cagemates was not demonstrated. The difficulty encountered in infecting rhesus monkeys despite the large virus dose employed indicates that this animal would not serve as a useful model for vaccine testing.

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Virus Carrier Cells and Virus-Free Cells in Fetal Rubella* (33348)

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Certain viral infections of animals occurring *in utero* are characterized by a persistence of the virus. In these animal models, the majority or totality of the cells may exhibit a state indicative of prior experience with the virus. Avian and murine leukemia viruses appear to be present in the majority of the cells of the host (1–3). Lymphocytic choriomeningitis virus (LCM) antigens are demonstrable in the majority of the cells of the early embryo of the mouse while relatively few cells of older animals contain viral antigen (4). *In vitro* studies have shown that previously infected cells which have become LCM antigen-negative are resistant to superinfection; this resistance to superinfection is not due to selection of insensitive clones of cells (5). In congenital rubella, however, it has been postulated that only a limited number of cells give rise to clones of persistently infected cells (6). The purpose of the present study was to directly determine the proportion of cells of the rubella-infected fetus which are producing virus and to see whether cells which do not produce virus exhibit evidence of prior experience with the virus.

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Materials and Methods. Tissue culture. Fibroblasts from lung tissues of fetuses from women not experiencing rubella, from normal newborn foreskins, and from skin and muscle tissue of fetuses exposed to rubella virus *in utero* were obtained by trypsinization with 0.25% trypsin (Difco 1:250) at 37° for 45–60 min. Fresh trypsin was added twice during this period. The cells derived from these sources were maintained in cultivation by several passages at 3–4-day intervals. The BSC-1 cells (7) and primary African green monkey kidney cells (GMK) (8) were cultivated and used for rubella virus assay. With the exception of GMK cells (8), all cells were grown in Eagle's basal medium supplemented with 10% fetal bovine serum; penicillin, 100 units/ml; kanamycin, 100 μg/ml; and 0.75 g/liter of sodium bicarbonate. Maintenance medium was the same except that 2% fetal bovine serum and 1.50 g/liter of sodium bicarbonate was used. Medium for cells grown in plastic petri dishes in a 5% CO_2 in air atmosphere contained 2.25 g/liter of sodium bicarbonate and nystatin (Mycostatin) 50 units/ml as an additional antibiotic. Agar overlay consisted of Eagle's basal medium, 10% fetal bovine serum, 2.25 g/liter of sodium bicarbonate, antibiotics, 1.2% Bacto-agar and 25 μg of neutral red/liter.

Viruses and virus assays. Rubella virus R-1, isolated from the thyroid of an infant with congenital rubella (6) was prepared in