A Variant of Parainfluenza Type 2 Virus (32852)

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In 1955 croup-associated (CA) virus was first isolated from children with acute laryngotracheobronchitis (croup) (1,2). In 1959 this virus was designated as parainfluenza type 2 virus (3). However, in previous studies of infections with type 2 parainfluenza virus (4–8), subtypes or antigenic variants distinguishable from the prototype Greer strain have not been isolated.

The present paper describes the isolation of two serologically different variants of parainfluenza type 2 virus from children with respiratory infections in Sendai. The first variant was identified serologically to be the same as the prototype Greer strain, but the second could be distinguished from the first as well as from the prototype Greer strain. The latter variant will be the main concern of this description.

Materials and Methods. Collection of specimens. Throat swabs were collected in 5 ml of Earle's balanced salt solution containing 0.5% lactalbumin hydrolyzate (Difco), 0.2% bovine serum (Difco), 500 units of penicillin, 500 μg of streptomycin and 50 units of nystatin/ml. Specimens were snap-frozen and stored at —70°C until use. Patients' sera were stored at —20°C.

Cell cultures. VERO cells established as a cell line by Dr. Y. Yasumura of Chiba University from green monkey kidney were used. The cells were grown in Earle's balanced salt solution with 0.5% lactalbumin hydrolyzate, 0.1% yeast extract (Difco) and 15% bovine serum. For virus isolation, Eagle's minimum essential medium containing 2% bovine serum (MEM) was used.

Virus isolation. Two-tenths ml of throat specimens were inoculated into each of 3 tubes of VERO cell cultures containing 1 ml of MEM. Tubes were rotated at 16 revolutions per hour at 33°C and the medium was exchanged on the fifth day. The cytopathic effect (CPE) was followed every day and hemadsorption test with guinea pig erythrocytes (9) was performed on day 10.

Hemagglutination test. Microtechnique (10) was employed for the test. Twofold serial dilutions were prepared in 0.05 ml of citrate saline (2.5% sodium citrate in saline, pH 7.2) with standard loops, and 0.05 ml of 0.6% suspension of either guinea pig or green monkey erythrocytes was added by dropper to each cup. The hemagglutinin titers were read after 2-hours' incubation at 36, 18, or 4°C.

Preparation of specific immune sera. Chicken and guinea pig antisera were prepared as follows: 10 ml of the infected cell culture fluid was injected intravenously into chickens which were bled 10 to 14 days later. Five-tenths ml of the same virus specimen was inoculated intranasally into guinea pigs under ether anesthesia and they were bled 3 weeks later.

Hemagglutination inhibition (HI) test. One part of serum and three parts of receptor destroying enzyme (RDE) were mixed and incubated at 37°C overnight. Twofold serial dilutions of the RDE-treated serum were prepared in 0.025 ml of citrate saline with standard loops, and 0.025 ml of virus containing 4 hemagglutinin units was added by dropper. After 1-hour incubation at room temperature, 0.05 ml of 0.6% suspension of the erythrocytes were added. The HI antibody titers were read after 2 hours' incubation at 36 or 4°C. For identification of the isolates, the following antisera were used: chicken sera for influenza A2/Adachi/57,A2/Kumamoto/64, B/Amakusa/64,B/Akishima/64,C/Yamagata/64, parainfluenza types 1 (C_{35}), 2 (Greer) and 3 (C_{243}), Sendai (Fushimi), SV5 (21005-2WR) and mumps (Enders) viruses; rabbit serum for Newcastle disease virus (NDV) and guinea pig sera for types 1, 2, and 3 parainfluenza, Sendai, SV5 and mumps viruses.

Neutralization test. Fourfold dilutions of inactivated serum were prepared with MEM without serum. Approximately 100 TCD_{50} of virus/0.1 ml was mixed with an equal

volume of the serum, and the mixture was placed for 1 hour at room temperature (20–25°C). One-tenth ml of the mixture was inoculated into VERO cell cultures and maintained on a roller drum at 33°C. On the sixth day of inoculation, on the basis of hemadsorption, 50% neutralizing antibody titers were calculated according to Reed and Muench. A simultaneous virus titration indicated that 100–320 TCD_{50} per 0.1 ml of virus was used in the test.

Complement-fixation (CF) test. The microtechnique was used for the test. All the sera were inactivated at 56°C for 30 min. Culture fluid of infected cell tubes was used as CF antigen.

Results. Virus isolation and passage. Three isolates of hemadsorption virus were recovered from two cases of respiratory infections employing VERO cell cultures, in January, 1966. The first, 1410 strain, was isolated from the throat swab of a 3-year-old girl with croup on the second day of illness. The other two isolates, 1430 and 1431, were obtained from the throat swabs of a one-year-old boy with upper respiratory illness, on days 11 and 12 of illness. These two cases lived in different districts of Sendai. In the VERO cell tubes inoculated with the above specimens, syncytia were demonstrated on the fifth to sixth day. All of the isolates could be passed in primary cynomolgus monkey kidney, VERO, HEp-2 cells and human diploid cells derived from embryonic kidney, but not in embryonated eggs. In the second passage of VERO cell cultures stained with hematoxylin and eosin, eosinophilic inclusion bodies were found in the cytoplasm of the syncytia but not in the nucleus.

Hemagglutinin of the isolate. Hemagglutinin titrations of the three isolates were performed at various temperatures using guinea pig and green monkey erythrocytes. As shown in Table I, the three isolates agglutinated both erythrocytes at 4°C and 18°C, but not at 36°C. However, it is known that prototype parainfluenza type 2 virus shows higher titers at 36°C than at 4°C with both erythrocytes.[1] Moreover, the 6 strains of type 2 parainfluenza virus recovered in October and November 1965, employing VERO cell cultures, agglutinated both erythrocytes at 36°C, and the titers were almost equivalent to or higher than those at 4°C, indicating the same biological characteristics as prototype 2 parainfluenza strain (Table II). Thus the results suggested that the three isolates of 1966 might be different from the other 6 parainfluenza isolates of 1965 as well as from prototype Greer strain.

[1] Numazaki, Y., unpublished data.

TABLE I. The Effect of Temperature on the Hemagglutination of 3 Parainfluenza Viruses Isolated in 1966.

Isolate no.	Guinea pig RBC			Green monkey RBC		
	4°C	18°C	36°C	4°C	18°C	36°C
1410	1:8	1:8	<1:4	1:16	1:16	<1:4
1430	1:16	1:16	<1:4	1:32	1:32	<1:4
1431	1:16	1:16	1:4	1:32	1:32	1:4

TABLE II. The Effect of Temperature on the Hemagglutination of 6 Parainfluenza Type 2 Viruses Isolated in 1965.

Isolate no.	Guinea pig RBC		Green monkey RBC	
	4°C	36°C	4°C	36°C
1258	1:16	1:16	1:16	1:16
1263	1:32	1:32	n.d.	1:64
1265	1:8	1:16	1:16	1:32
1275	1:8	1:16	1:32	1:64
1279	1:16	1:32	n.d.	1:64
1330	1:16	1:32	n.d.	1:32
Greer	1:8	1:16	1:16	1:32

Identification of isolates as parainfluenza type 2 viruses. The HI test was employed for the typing of the three isolates of 1966. However, the three isolates were shown not to be inhibited by any of the antisera prepared against myxoviruses, including parainfluenza type 2, but were inhibited by the one prepared against isolate 1430 as shown in Table III. The guinea pig antiserum prepared against the 1430 strain was equally potent against the Greer strain of parainfluenza type 2 virus and the three new isolates of 1966. Other prototype viruses tested were not inhibited.

The CF test was also employed for the identification of the three isolates using guinea pig antisera prepared against parainfluenza types 1, 2, and 3; Sendai; SV5; and

TABLE III. Typing of Three Isolates of 1966 by HI Test Employing Chicken Immune Sera.

Virus	HI antibody titer of the antisera prepared against:											
	Para 1	Para 2	Para 3	Sendai	SV5	Mumps	NDV[b]	A2/57	A2/64	B/64	C/64	1430[c]
Para 1	256	0[a]	0	0	0	0	0	0	0	0	0	0
Para 2	0	256	0	0	0	0	0	0	0	0	0	128
Para 3	0	0	256	0	0	0	0	0	0	0	0	0
Sendai	0	0	0	128	0	0	0	0	0	0	0	0
SV5	0	0	0	0	256	0	0	0	0	0	0	0
1430	0	0	0	0	0	0	0	0	0	0	0	128
1431	0	0	0	0	0	0	0	0	0	0	0	128
1410	0	0	0	0	0	0	0	0	0	0	0	128

[a] 0 = less than 1:32.
[b] Rabbit serum.
[c] Guinea pig serum.

mumps viruses. Only the antiserum prepared against type 2 Greer strain fixed complement in the presence of undiluted culture fluid infected with the three isolates.

Antigenic relationship between the three isolates of 1966 and the prototype Greer strain of parainfluenza type 2 virus. The HI tests of the Greer strain, 6 parainfluenza 2 isolates in 1965 and 3 isolates in 1966 were performed employing both the chicken and guinea pig immune sera made against the Greer strain. As shown in Table IV, the three strains in 1966 were inhibited specifically by anti-1430 serum, but not by anti-Greer sera. However, the 6 isolates in 1965 as well as the Greer strain were inhibited by both anti-Greer and anti-1430 sera. Anti-1430 chicken serum was prepared but the titer was inadequate for study.

TABLE IV. HI Test of 10 Isolates with Specific Guinea Pig and Chicken Sera against Greer and 1430 Strains.

Isolate no.	Anti-Greer		Anti-1430 guinea pig
	Chicken	Guinea pig	
1410	<1:32	<1:32	1:128
1430	<1:32	<1:32	1:128
1431	<1:32	<1:32	1:128
1258	1:128	1:256	1:128
1263	1:256	1:128	1:256
1265	1:512	1:128	1:256
1275	1:256	1:256	1:128
1279	1:512	1:128	1:128
1330	1:128	1:128	1:128
Greer	1:512	1:256	1:128

TABLE V. Antigenic Relationship between 1430 and Greer Strains by HI Test with Small Hemagglutinins.

Virus (treatment)	Anti-Greer		Anti-1430 guinea pig
	Chicken	Guinea pig	
Greer (untreated)	1:512	1:256	1:128
1430 (untreated)	<1:32	<1:32	1:128
1430 (sonicated)	<1:32	1:32	1:128
1430 (ether-disrupted)	1:1024	1:512	1:128

The viral hemagglutinin of the 1430 strain was further treated by sonication and ether in order to prepare the small hemagglutinins (11,12). When whole cultures of infected VERO cells were sonicated for 15 min at 10 kc/sec, hemagglutinin titers increased 4-fold after the sonication. For obtaining ether-disrupted hemagglutinins, equal parts of the infected culture fluid and ether were mixed and incubated for 30 min at 4°C with repeated shaking. The mixture was centrifuged for 10 min at 2000 rpm. The ether phase was drawn off and the residual ether was allowed to evaporate. The resulting hemagglutinins revealed a 2-fold increase in titer, and the titer was the same both at 36°C and 4°C. The HI tests were then performed using the 3 types of hemagglutinin preparation against the above sera. As shown in Table V, ether-disrupted small hemagglutinins were inhibited by

TABLE VI. Antigenic Relationship between 1430 and Greer Strains by Neutralization and Complement Fixation Tests.

Virus	Anti-Greer			Anti-1430 guinea pig	
	Chicken	Guinea pig			
	neut.	Neut.	CF	Neut.	CF
Greer	1:32	1:724	1:64	1:91	1:64
1430	1:4	1:182	1:64	1:182	1:64

both anti-Greer and anti-1430 sera, whereas the sonicated as well as untreated hemagglutinins were not inhibited by anti-Greer but inhibited by anti-1430 sera. Thus it is suggested that ether-disrupted small hemagglutinin of the 1430 strain shares a common antigen with the Greer strain, but this antigen might not be exposed in the untreated or the sonicated hemagglutinins.

The serological properties of Greer and 1430 strains were also compared using the neutralization test. Neutralizing antibody titers against the 1430 strain were significantly lower than those against Greer strain when anti-Greer serum was used, but there was no difference between Greer and 1430 strains when anti-1430 serum was used for the neutralization (Table VI). In cross CF tests, there was no difference between 1430 and Greer strains.

Antibody response of patients from whom the three isolates were recovered. The HI tests were performed using sera of the two patients from whom the three isolates were recovered. Although there was no significant antibody rise against prototype strains of parainfluenza types, 1, 2, and 3; Sendai; SV5; mumps; and influenza viruses, HI antibody rise against 1430 strain was significant in convalescent sera as shown in Table VII.

Discussion. Before 1965, all of the parainfluenza type 2 isolates in Sendai were not distinguishable from the prototype strain Greer in hemagglutination-inhibition test. However, the three isolates recovered from two children with respiratory illness in January 1966 were differentiated from the prototype Greer strain of parainfluenza type 2 virus using the following criteria: (i) These strains did not readily agglutinate guinea pig or green monkey erythrocytes at 36°C. This characteristic may be contrasted to those of the prototype strain and other isolates of parainfluenza type 2 virus, which showed higher titer at 36°C than 4°C with both erythrocytes. (ii) Although the hemagglutinins of the three isolates were not inhibited by any of the myxovirus antisera including the serum against type 2 parainfluenza virus, the three isolates fixed complement with antiserum prepared against the Greer strain of parainfluenza type 2 virus. (iii) The guinea pig immune serum prepared against the 1430 strain inhibited specifically the hemagglutinin of the Greer strain as well as homologous strain. Thus the three isolates were thought to be included in the parainfluenza 2 serotype.

The antigenic relationship between the three newly isolated strains and the prototype Greer strain of parainfluenza type 2 virus was therefore investigated. Six strains of type 2 parainfluenza virus recovered in Sendai in 1965 were also investigated in a comparative manner. As described above, there is a classical "one-way cross" between the 1430 strain

TABLE VII. HI Antibody Response to Myxoviruses in Paired Sera of Patients from Whom 3 Isolates Were Recovered.

Case no.	Day of illness	HI antibody titers against:									
		Para 1	Para 2		Para 3	Sendai	SV5	Mumps	A2/64	B/64	C/64
			Greer	1430							
2452	2	0[a]	0	0	0	32	0	0	0	0	0
	21	0	0	128	0	32	0	0	0	0	0
2290	11	0	0	32	64	64	0	0	0	0	0
	25	0	0	128	64	32	0	0	0	0	0

[a] 0 = less than 1:32.

and the prototype strain in the HI test.

However, when the ether-disrupted small hemagglutinin of 1430 strain was used, it was inhibited by both anti-Greer and anti-1430 sera. Taken together, it is suggested that a 1430 variant contains an antigen in common with the prototype, but that it is either not exposed to the surface or is blocked by an inhibitor from reacting with its antibody. This is indicated by the demonstration that when the 1430 strain is used as an immunizing antigen, antibody is made to this "hidden component." The finding that sonication yields this component poorly or not at all, as shown in Table V, while ether yields a high titer of this component, may simply reflect the fact that sonication acts largely in disaggregating whole virions, while ether serves to split the virus into small hemagglutinin components or possibly to remove an inhibitory substance. The inability of the new isolates to agglutinate erythrocytes to high titer at the higher temperature suggests a difference in neuraminidase or other physical activity of the virions that might incidentally account for differing avidity with antibody.

A partial one-way cross is also demonstrated in the neutralization test, which would be compatible with the concept of nonavailability of the antigen at the surface. The CF test crosses completely, which represents either crossing of soluble antigen or a broad reactivity of the envelope protein between the two variants.

On the basis of the HI test and, to a lesser extent, the neutralization test, two variants of parainfluenza type 2 virus were demonstrated in the present paper. The present findings represent the first demonstration of antigenic variation among naturally occurring strains of type 2 virus isolated in the same VERO cell cultures, although a subtype of parainfluenza type 4 virus has been reported in 1964 (13). The unique feature of the parainfluenza 2 variant, which is based on a one-way cross, is the demonstration of a hidden common hemagglutinin antigen following disruption of the particle with ether.

The three new strains were isolated employing VERO cell cultures. In addition, HI antibody response against the newly isolated 1430 occurred in paired sera obtained from patients from whom viruses were recovered. These features appear to rule out the possibility that the new isolates are contaminated with simian hemadsorption virus. Whether the new variant will replace the prototype as the predominant organism in future epidemics remains to be determined.

Summary. Three isolates of hemadsorption virus were recovered from children with respiratory infections in January 1966. The three isolates could not be distinguished from the prototype Greer strain of parainfluenza type 2 virus by the complement-fixation test using antisera against both the new isolate and Greer strain. However, the neutralization test and the hemagglutination-inhibition test revealed a difference between the new isolates and the prototype Greer strain. The antiserum against Greer strain was not inhibitory for the new isolates in the hemagglutination-inhibition test of the latter viruses before and after sonication and also in the neutralization test, whereas ether disruption of the hemagglutinin disclosed a common antigen in the new isolates and the Greer strain. In contrast to this, 6 isolates of 1965 were the same as the Greer strain in all serological tests.

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