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INTRATYPIC HETEROGENEITY IN ADENOVIRUS TYPE 3*

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SUMMARY The heterogeneity of 7 strains of freshly isolated type 3 adenovirus to prototype strain (G.B.) was examined by cross neutralization tests using naturally convalescent children's sera and guinea pig immune sera. From the results, the 8 strains were divided into 4 groups. There were serological differences between prototype (G.B.) and freshly isolated Tokushima 65, Osaka 64, Tokyo 63 and Hyogo 63 strains (groups C and D). Less heterogeneity was observed between Ehime 64 and Osaka 65 strains (group B) and the prototype, than in groups C and D. Tokyo 56 strain was serologically similar to prototype (G.B.).

INTRODUCTION

Type 3 adenoviruses have been shown to exist in nearly all parts of the world (HUEBNER *et al.*, 1958., ROWE *et al.*, 1956). Sporadic outbreaks of acute respiratory disease caused by adenovirus type 3 (adeno t. 3) have been identified in many children, particularly in schools and summer camps (HILLMAN *et al.*, 1959., FUKUMI *et al.*, 1958., MUTAI *et al.*, 1965). In summer, 1964, outbreaks of acute respiratory illness due to adeno t. 3 occurred at Ikeda city in Osaka, Japan.

The amount of neutralization antibody increased in sera from convalescent patients more with freshly isolated Osaka 64 than with prototype (G.B.). This suggested that there was serological heterogeneity between prototype (G.B.) and freshly isolated Osaka 64 strain. Thus heterogeneity between the prototype and other freshly isolated strains of adeno t. 3 could be anticipated.

Cross neutralization tests with 3 groups of convalescent children's sera and guinea pig immune sera against the G.B. strain and other freshly isolated strains also indicated the presence of intratypic heterogeneity among various strains of type 3 adenovirus.

Several workers have reported heterogeneity between the prototypes and " prime " types of various adenoviruses. For instance, a serologic difference between type 7 and 7a can be distinguished by the neutralization test, but not by the hemagglutination inhibition (HI) test (ROWE *et al.*, 1958). On the contrary, " prime " strains of types 16, 17 and 27 have been identified by the HI test, although the " prime " relationship is not revealed by the neutralization test (ROSEN, 1962).

An intermediate type of types 3 and 7, which is neutralized by both type 3 and type 7, was

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identified as type 3 by the hemagglutination inhibition procedure (MUTAMOTO *et al.*, 1958., ROSEN, 1960).

We do not know of any reports on intratypic heterogeneity in adenovirus type 3. In the present work, the heterogeneity between prototype (G.B.) and freshly isolated strains of adenovirus type 3 was studied by the cross neutralization test.

MATERIALS AND METHODS

1. *Cells and tissue cultures*

KB cells were employed throughout. KB cells were propagated in bottles (50×110mm, 150×220mm) in growth medium consisting of YLE (Earle's balanced salt solution containing 0.1% yeast extract and 0.5% lactoalbumin hydrolysate) containing 10 per cent inactivated bovine serum. When monolayers became confluent, they were dispersed and propagated in roller tubes (10×150 mm, 15×10^4 cells/ml) with growth medium, which was replaced by maintenance medium (YLE supplemented with 2 per cent inactivated horse serum) after 2 days for neutralization tests. All the above media contained 100 units of penicillin and 100 μg of streptomycin per ml.

2. *Viruses and virus preparations*

Prototype (G.B.) and 7 freshly isolated strains of adenovirus type 3 were employed. The freshly isolated preparations are described in Table 1. All the strains were obtained by inoculating tissue culture with throat washings taken from patients diagnosed as having an acute respiratory disease. The prototype (G.B., pool 194) and Tokyo 56 strain were kindly given by Dr. NAKANO (National Institute of Health, Tokyo, Japan).

Confluent KB monolayers were inoculated with adenovirus strains. The cultures were observed daily, and cells were harvested from all bottles when complete cytopathic effects were observed and were pooled. The harvested cells and fluid were freeze-thawed 3 times and then centrifuged for 10 minutes at 3,000 rpm. The supernatant fluid was dispersed in approximately 2 ml volumes in glass ampules. These were sealed, rapidly frozen and stored at —20°C.

Not all freshly isolated samples were intermediate types of type 3 and type 7. The prototype strain (G.B.), which had been highly passaged in our laboratory, was employed mainly in the experiments, since there was no serological difference in cross neutralization tests between the G.B. strain of our laboratory and G.B. (HL6 KB4 HL1) obtained from N.I.H, Japan.

3. *Sera*

A) *Human sera*

Sera from 54 convalescent children of " S " elementary school in Ikeda, Osaka, were an outbreak of acute respiratory illness of the adenovirus type 3 occurred in 1964, were employed. Sera from 43 children of " I & K " elementary school in Ikeda, suffering from pharyngoconjunctival fever in 1959, were also used.

3 groups of adenovirus type 3 sera were obtained from 12 convalescent children who had suffered from pharyngo-conjunctival fever in Tokyo, in 1963, 9 children in Osaka, in 1964, and 7 children in Tokushima, in 1965.

TABLE 1 *Strains of Adenovirus Type 3 used*

Strains of adenovirus type 3	Date of virus isolation	Passage history	Geographic origin
prototype (G.B.) freshly isolated	1953	HL6 KB4 HL1	Washington, D.C.
Tokyo 56	1956	HL1 KB5	Tokyo, Japan
Tokyo 63	1963	HL2 KB5	Tokyo, Japan
Hyogo 63	1963	HL2 KB6	Hyogo, Japan
Ehime 64	1964	HL1 KB5	Ehime, Japan
Osaka 64	1964	HL1 KB5	Osaka, Japan
Tokushima 65	1965	HL2 KB6	Tokushima, Japan
Osaka 65	1965	KB6	Osaka, Japan

B) *Immune sera*

Sera of the 8 strains described in Table 1 were prepared using guinea pigs. Guinea pigs were given one intraperitoneal injection of live virus (approximately 2×10^6 $TCID_{50}$/0.1 ml) and bled 4 weeks later.

C) *Hyperimmune sera*

Rabbits were given two intramuscular injections of crude virus in adjuvant at weekly intervals followed, a week later, by one intraperitoneal injection live virus without adjuvant. Animals were bled 10 days after the last injection.

The sera were separated by centrigugation, inactivated by heating at 56°C for 30 minutes, and stored at −20°C.

4 Neutralization test

This test was carried out in a KB cell culture. The maintenance medium was YLE containing 2% inactivated horse serum. Virus was diluted in Hanks' solution so that 0.1 ml of solution contained just enough virus to destroy the KB cells in the test tubes completely (3+) in 3 days. All sera were inactivated at 56°C for 30 minutes prior to use and 2 fold serially diluted with Hanks' solution.

One volume of diluted virus sample and one volume of diluted serum were mixed and the mixture was kept at 37°C for 30 minutes and overnight (TOYOSHIMA *et al.*, 1965). Then 8 volumes of medium were added. Volumes of 1 ml of the mixture were then transfered to two KB cell cultures.

As controls, 2 tubes of cell culture inoculated with the diluted virus mixed with Hanks' solution instead of serum were included. Results were estimated after complete cytopathogenic effect had appeared in the control tubes.

Neutralization titers are expressed as the reciprocal the highest dilution of antiserum causing complete inhibition of virus CPE (UCHIDA *et al.*, 1959).

RESULTS

1. *Serologic Heterogeneity between Prototype (G.B.) and freshly isolated Osaka 64 Strain with 2 Groups of Sera*

Investigations on the antigenic difference between prototype (G.B.) and Osaka 64 strain were performed using 2 groups of human convalescent sera. Convalescent sera from schoolboys of "S" elementary school who had suffered from acute respiratory diseases due to adenovirus type 3 in 1964, and sera of schoolboys of "I & K" elementary school infected with adenovirus type 3 in 1959 were employed in neutralization tests.

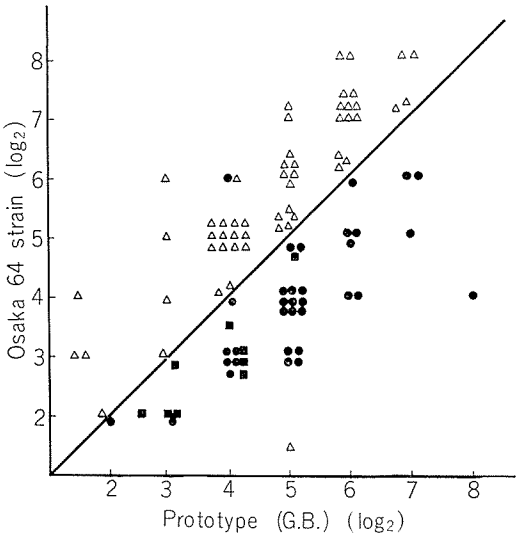


FIGURE 1 Relationship between neutralizing antibody titers of sera of children infected in 1964 ("S" School; △) and sera of children infected in 1959 ("I & K" school; ●, ■) against prototype (G.B) and Oska 64 Strain.

The 2 groups of sera were titrated against prototype (G.B.) and Osaka 64 strain.

The graphic presentation of the relationship of the neutralization titers of the 2 groups of sera against G.B. strain and Osaka 64 strain is shown in Fig. 1.

From the results, Osaka 64 strain is easily neutralized by sera of boys of "S" school, but prototype (G.B.) is not 'apparently. In sera of boys of "I & K" school, G.B. strain is neutralized more than Osaka 64.

It was found that there was about 4 fold difference between sera of boys of "S" school and "I & K" school.

A highly significant difference, $P<0.001$, was observed between the neutralizing antibody of sera of boys from "S" and "I & K"

school. Therefore, there is an apparent antigenic difference between prototype (G.B.) and Osaka 64 strain.

2. *Relationship between Prototype* (G.B.) *and various freshly isolated Virus Samples in Cross Neutralization Tests using* 3 *Groups of convalescent Sera*

Fig. 1 shows that freshly isolated Osaka 64 has serologic heterogeneity to the G.B. strain, prototype.

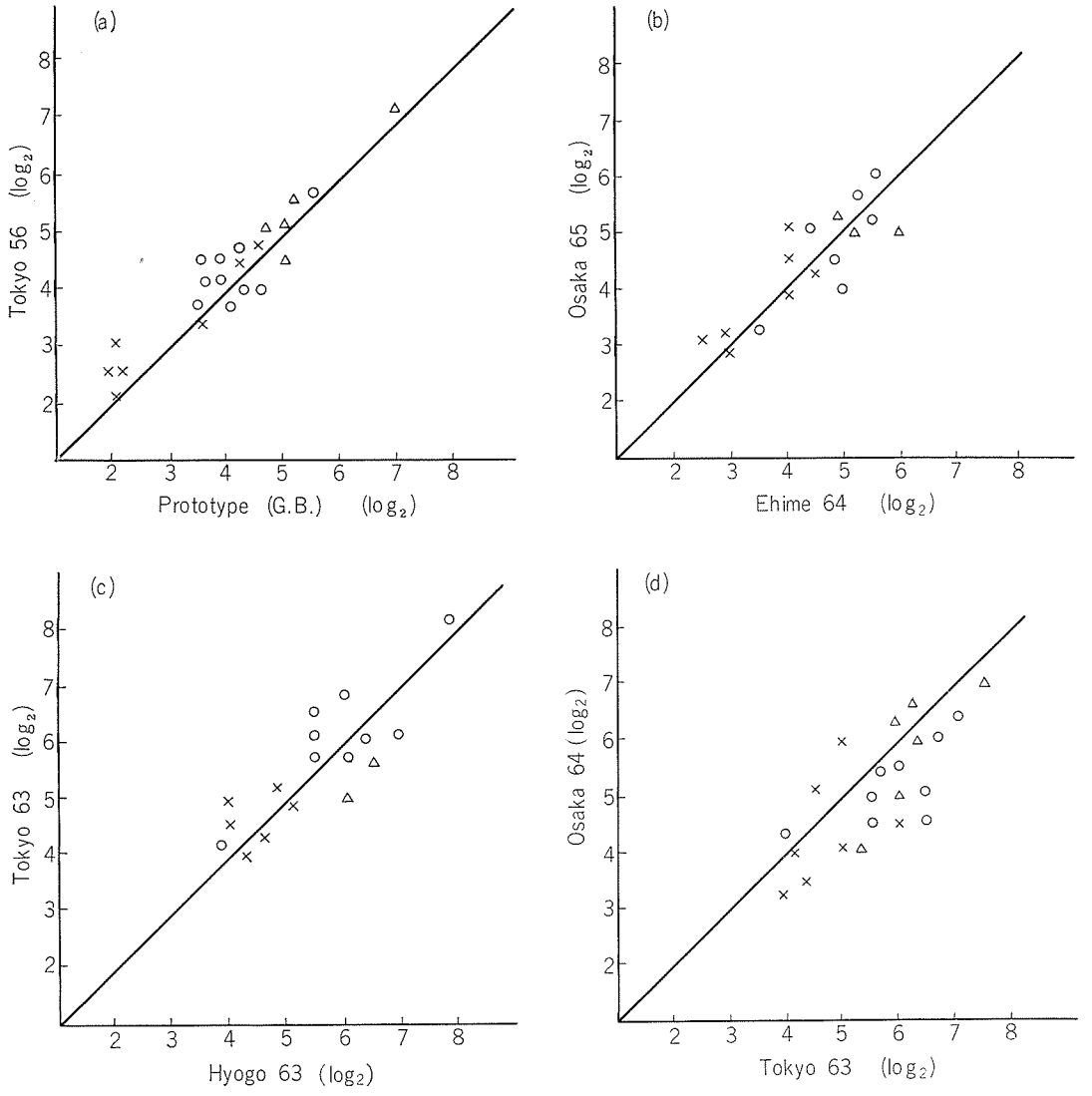


FIGURE 2 (a)～(j) Relationship of 8 strains in the cross neutralization test using 3 groups of convalescent sera.

Convalescent sera after infection with adenovirus type 3.

○, in Tokyo, 1963; ×, in Osaka, 1964; △, in Tokushima, 1965.

In the following experiments, the possibility that other freshly isolated viruses could have antigenic heterogeneity against G.B. strain was tested.

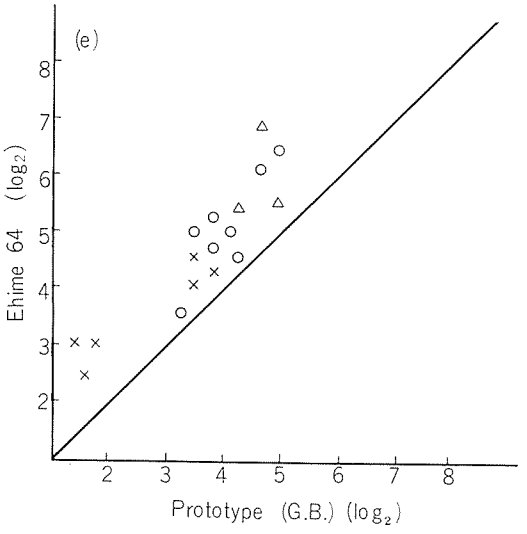
The freshly isolated strains used in the experiments were Tokushima 65, Osaka 65, Ehime 64, Osaka 64, Tokyo 63, Hyogo 63, Tokyo 56 and prototype (G.B.). 3 groups of sera from convalescent patients infected with adenovirus type 3 were employed.

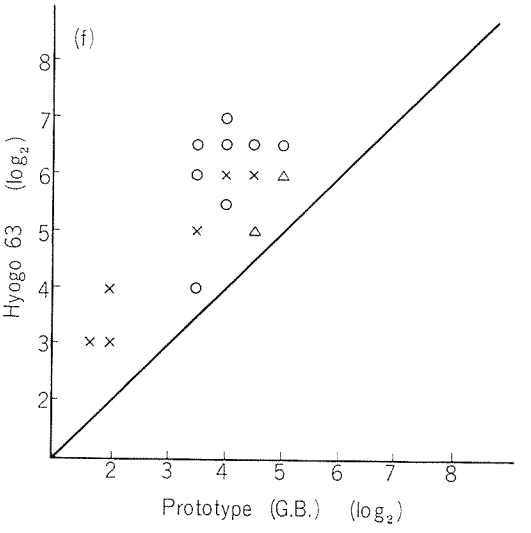
The convalescent sera against 8 strains were titrated by the neutralization tests.

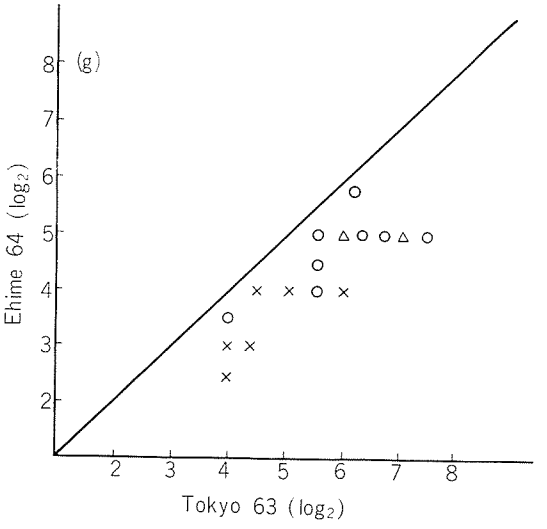
Fig. 2 (a)~(j) represent the relationship between 2 representative strains by the neutraling antibody titers.

Fig. 2 (a) shows that prototype (G.B.) and Tokyo 56 are close together in the middle of the figure. This indicates that the two strains are closely related antigenically.

In Fig. 2(c), the relationship between Tokyo







63 and Hyogo 63 is shown. From Fig. 2(c)～(d), strains Tokyo 63, Hyogo 63 and Osaka 64 are very similar antigenically.

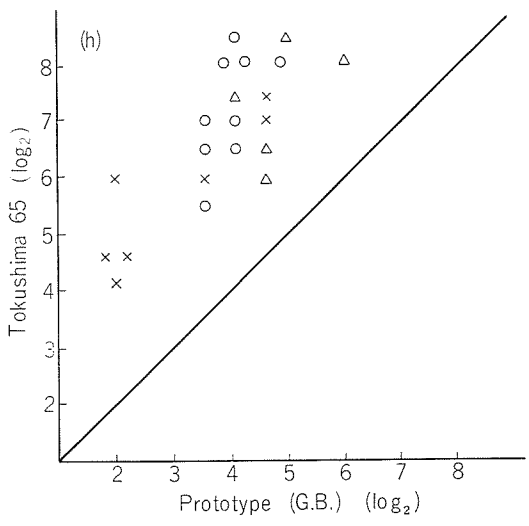
Fig. 2(a)～(d) indicates that the 7 strains other than the Tokushima 65 strain can be divided into 3 groups; i.e,

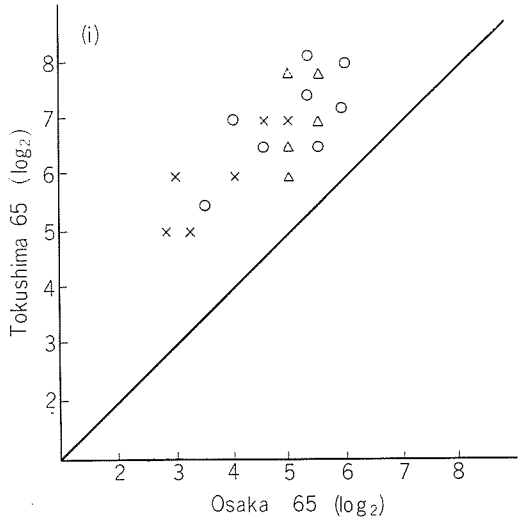
A) prototype (G.B.) and Tokyo 56 strains
B) Ehime 64 and Osaka 65 strains
C) Tokyo 63, Hyogo 63 and Osaka 64 strains

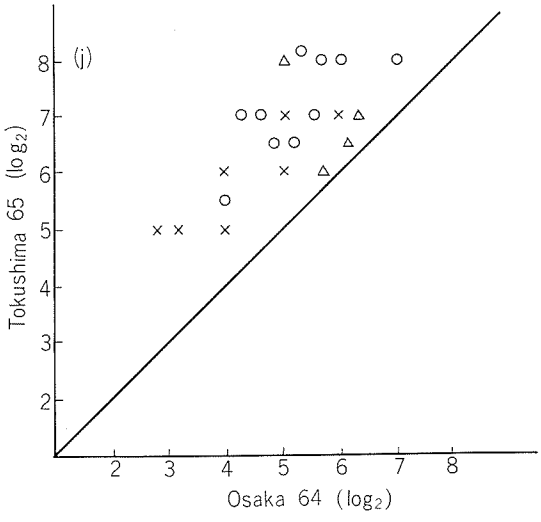
Fig. 2(e)～(g) represent the correlation of Tokushima 65 with the 3 groups of strains. According to the figures, Tokushima 65 strain is easily neutralizable with the sera of patients.

This indicates that Tokushima 65 is different from the 3 groups of strains. Tokushima 65 strain was assigned to a group D.

Fig. 2(h)～(j) shows the relationship between the 4 groups of strains. From the figures,







the 4 groups of strains are graded ;

A)→B)→C)→D) groups

It was found that Tokushima 65 strain has the highest neutralizability with prototype (G.B.).

3. *Heterogeneity between Prototype (G.B.) and freshly isolated Viruses in Guinea Pig Immune Sera*

8 strains of adenovirus type 3 were immunized using guinea pigs to confirm the above results.

Cross neutralization tests with the 8 strains and guinea pig immune sera were performed.

The results are shown in Table 2. From the cross neutralizations of group B), C) and D) antisera, strains of group A) were given much lower titers than those to other strains,

TABLE 2 *Reciprocal neutralization tests of prototype (G.B.) and 7 freshly isolated viruses with guinea pig antisera*

	Viruses	Guinea pig immune sera												
		Prototype		Tokyo 56	Ehime 64		Osaka 65	Tokyo 63		Hyogo 63		Osaka 64	Tokushima 65	
		#1	#2		#1	#1		#1	#2	#1	#2		#1	#2
A)	Prototype (G.B.)	7	3	≦2	3.5	4	2.5	≦2	2.5	3	3	2.5	≦2	≦2
	Tokyo 56	6.5	3	3.5	3.5	4	2.5	2.5	3	3	3.5	3.5	≦2	≦2
B)	Ehime 64	5.5	2	2.5	5	6	3.5	3.5	4.5	4	4.5	5.5	4.5	3
	Osaka 65	5.5	2	3	5	6.5	4.5	4	4.5	4.5	5	5	5	3
C)	Tokyo 63	6	3	4	6	6.5	5	5	6.5	6	5.5	7.5	7	5
	Hyogo 63	6.5	3	3.5	6	7	5	5.5	6.5	6	6	7	6	5
	Osaka 64	6	3	4	6	7	4.5	5	6	6	6	6	5.5	4
D)	Tokushima 65	6	3	4.5	6	6.5	5	5	7	5	6	7	6	5

Figures show neutralizing antibody titer (Log_2)

TABLE 3 *Neutralization tests using hyperimmune rabbit antisera*

Viruses	Rabbit hyperimmune sera		
	Prototype	Tokyo 63	Osaka 64
Prototype (G.B.)	11	9	9
Tokyo 56	11	9	9.5
Ehime 64	N-T	N-T	N-T
Osaka 65	N-T	N-T	N-T
Tokyo 63	10	10	10
Hyogo 63	N-T	N-T	N-T
Osaka 64	9	9.5	10
Tokushima 65	N-T	N-T	N-T

Figures show neutralization titer (Log_2)
N-T : not tested

but higher antiserum titers against C) and D) strains were given differing by 4 to 16 bold than the antibody titers to prototype (G.B.).

From the results on group A) antisera, the antibody titers determined with A) strains are nearly as high as those with the other strains. Thus, no significant difference in neutralizing antibody titers was observed.

It is shown in Table 2 that the serological heterogeneity of C) and D) strains to G.B. strain is more apparent than the results presented in Fig. 2(a)～(j), although no clear difference between the C) and D) strains is shown.

Table 3 shows the results of neutralization tests using rabbit hyperimmune sera. The results show that there was no difference in

the neutralization titers against the various strains.

All the experiments described were reproducible.

In the hemagglutination inhibition test, no apparent heterogeneity was seen between the prototype and the other strains isolated.

DISCUSSION

This report describes intratypic heterogeneity in adenovirus type 3. 8 strains were obtained and divided into 4 groups.

4 strains (Tokushima 65, Hyogo 63, Osaka 64 and Tokyo 63) freshly isolated in our laboratory had apparent serological differences from prototype (G.B.).

Immunization experiments (Table 1) suggested one way cross reaction of the G.B. strain with the 4 strains of the C) and D) groups.

" S " elementary school has many children who have recently moved to Ikeda city, Osaka, but " I " school in the neighborhood of " K " school is situated in an area of the city where the inhabitants have scarcely changed. Accordingly, it was thought that the strain which caused widespread infection of boys of " S " school in 1964 differed from the G.B. strain antigenically, while strains closely related to the G.B. strain caused outbreaks of acute respiratory diseases at " I " school in 1959.

Two possibilities may be considered.

First, viruses similar to the G.B. were prevalent in Ikeda city from 1959 to 1961, and then, strains closely related to the Osaka 64 strain became dominant. Second, the two strains might coexist in one area, and both might cause widespreads of acute respiratory diseases.

There was no serological difference between the 6 strains isolated from 1963 to 1965, but prototype (G.B.) and Tokyo 56, isolated approximately 10 years previously, represented antigenic variants from groups C) and D) of strains. It is thought that adenovirus type 3 has gradually changed over about ten years from 1953.

Group D) of Tokushima 65 strain, which responded broadly to various antisera, might be useful for diagnostic surveys.

It seems likely that adenovirus type 3 tends to develop a comparatively small area, since it did not cause pandemic outbreaks of disease.

Sporadic outbreaks of adenoviruses each year, may be due to antigenic variations of virus, as is known to be the case with influenza virus.

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