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Antigenic Analysis of Type I Poliovirus Analysis of the Recent Epidemic Strains in Japan*

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SUMMARY

An epidemiological analysis of wild type 1 poliovirus was made using the kinetic neutralization test. The large epidemics which occurred in Hokkaido and Kyushu, Japan in 1960 and in 1961 were caused by serologically homologous viruses. In the large cities in the central part of Japan, there was no large epidemic and viruses both homologus and heterologous to the epidemic group were isolated during 1960 and 1961. Epidemics before 1960 were caused by viruses serologically heterologous to the recent epidemic group.

For these analyses small but reproducible differences in normalized K values were utilized to discriminate between the heterologous strains. To confirm the heterogenicity of these strains, the absorption test of serum with active virus was employed and the usefulness of this test for differentiation of small differences in antigenicity was established.

INTRODUCTION

Antigenic valiability among strains within a given poliovirus type was first noted by Wenner *et al.* using the end-point neutralization test (1956, 1959). On the other hand the kinetics of the serum neutralization test of poliovirus was shown by Dulbecco *et al.* (1956) and this technique was applied to accurate analysis of intratypic strains of this virus by McBride (1959). He found that heterologous strains were neutralized more slowly than the homologous strain and strains isolated from the same general area in the same year appeared to be more similar antigenically than others. Subsequently, three other serological techniques were introduced by Wecker (1960), Gard (1960) and Melnick and Benyesh-Melnick (1960). Gard intended to confirm Wenner's technique and to test whether a given strain of virus was derived from a certain source of infection. The technique was confirmed and extended by his work, but no correlation was found between infective sources. These five techniques were applied for surveillance of the progeny of live poliovirus vaccines and were found to be useful for analysis of accidental paralysis after administration of oral vaccines (Koprowski *et al.*, 1960; Plotkin *et al.*, 1961; Ozaki *et al.*, 1963; Diwan *et al.*, 1963; Toyoshima *et al.*, 1963). On

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the other hand, the epidemiology of wild poliovirus has seldom been analyzed by these techniques.

Most of the polio-epidemics in Japan were caused by type 1 virus (Kono, 1960) and a large epidemic caused by type 1 virus was observed recently. Therefore type 1 virus strains isolated in various districts of Japan between 1956 and 1961 were tested. In our laboratory the intratypic serodifferentiation test by the kinetic neutralization technique (McBride, 1959) has been applied to antigenic analysis of poliovirus, because this test is independent of the growth rate of the virus. This report includes epidemiological analysis of type 1 poliovirus strains, especially those isolated during epidemics in 1960 and 1961.

MATERIALS AND METHODS

1. *Viruses*

Mahoney, Brunenders, LSc 2ab and Lederle SM strains were employed as standard laboratory strains for the test. The former 3 strains were tested after 2 plaque passages. Wild type 1 strains were kindly supplied from many laboratories (Tables 1 and 2) and 112 out of the 158 strains were employed for the tests after single passage in monkey kidney tissue culture in our laboratory. Virus stocks were harvested in medium 199 and stored at −20°C.

2. *Anti-sera*

Anti-Mahoney, anti-LSc 2ab, anti-Lederle SM, anti-Ogawa (wild, Osaka, 1961), anti-Kawasaki (wild, Hokkaido, 1959), anti-Y-12-61 (wild, Yamaguchi, 1961) and anti-Hirasawa (wild, Tokyo, 1959) sera were prepared by the following schedule: Stock virus pools were partially purified by fluorocarbon treatment and about 10^9 PFU of each strain were inoculated subcutaneous-

Table I. Virus Sources

Number of strains	Supplied by	
11	M. Kanamitsu	Sapporo Medical College
26	Y. Hinuma	Tohoku University
28	N. Nakano	National Institute of Health, Japan
16	K. Ishii	Ebara Hospital, Tokyo
6	M. Hirayama	The University of Tokyo
15	R. Kono,* Y. Ashihara* and C. Hamada	Kyoto University
16	E. Majima and S. Okajima	Osaka University
14	K. Kawakami	Osaka Medical College
11	T. Hotta	Institute of Health, Osaka City
7	M. Shingu	Kurume University
7		Osaka Public Health Institute

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Table 2. Geographic Distribution of Wild Strains

Geographic source		Year of isolation					
		1956	1957	1958	1959	1960	1961
Northern part	Hokkaido	3		3	1	5	
	Tohoku				26	2	
Central part	Kanto *		2	8	10	9	2
	Chubu			1	1		
	Kinki**	4	7	13	13	10	7
Western part	Shikoku	2	1				
	Chugoku		6	2			2
	Kyushu			8	4	3	3

* Most strains were isolated in Tokyo.
** Most Strains were isolated in Osaka.

ly into 2 to 4 rabbits. Two or three booster doses (10^8 PFU) were given intracutaneously at 3 week intervals and 7 days after the last immunization rabbits were bled by heart puncture. Anti-sera were tested by kinetic neutralization with isologous and a heterologous strain and selected for neutralization experiments. Anti-Brunenders serum was kindly supplied by the Research Foundation for Microbial Diseases, Osaka University.

3. *Virus assay*

All viruses were assayed by the plaque method with half log dilutions of virus.

4. *Tissue culture*

Primary monkey kidney tissue cultures were prepared by Bodian's method and employed throughout the experiments. Five to 6 days after seeding the cells, plaque bottles were washed once with phosphate buffered saline (PBS) and then 7 ml of Earle's saline supplemented with 0.5 per cent lactalbumin hydrolysate and 0.2 per cent skimmed milk was introduced into the bottles. These bottles were used on the following day for virus assays or virus growth.

5. *Neutralization test*

The kinetic neutralization test was performed according to McBride's method. The anti-serum was diluted with PBS to a dilution which showed a neutralizing capacity to reduce the infective titer of homologous virus to $10^{-1}-10^{-2}$ in 10 min. at 37°C. Stock viruses were diluted to approximately 5×10^6 PFU per ml of virus. These were placed in a water bath at 37°C and the diluted serum was distributed in 0.5 ml amounts into 10×75 mm tubes. Equal volumes of virus was added to the tubes at time zero and 0.1 ml of the virus-serum mixture was transfered into 50 ml cold PBS at 5 minutes intervals. The residual virus titer of each sample was determined by plaque titration and the results of neutralization tests are expressed as the average of the normalized K values (NK value) of serveral experiments.

$$K = \frac{D \log e}{t} \cdot \log \frac{Vo}{Vt} \qquad D\text{: dilution of serum, } t\text{: time in min.}$$

Vo; virus titer at time zero, Vt; residual titer at time t.

$$NK = \frac{K \text{ of each strain}}{K \text{ of isologous strain}} \times 100$$

6. *Absorption test of serum*

Serum was diluted to half the dilution employed for the kinetic neutralization test. The serum was distributed in test tubes in 6 ml aliquots and mixed with equal volumes of active virus samples containing about 10^7 PFU/ml. After incubation at 37°C, for 3 hours, mixtures were centrifuged at

105,000 × g for 90 minutes. The supernatant fluids were heated at 56°C for 30 minutes. The control serum for this experiment was treated in the same manner, but Hanks' balanced salts solution was employed rather than virus material.

RESULTS

Preliminary tests on the antigenic specificity of wild strains

Antigenic similarities of type 1 poliovirus strains isolated in 1961 were demonstrated in the previous report (Toyoshima *et al.*). To test whether the many strains isolated in Japan were also similar, kinetic curves were drawn with anti-Ogawa (Osaka, 1961), anti-Mahoney and anti-Brunenders sera. As seen in Table 3, both antigenically closely related and considerably different strains were observed with anti-Ogawa serum. Ogawa homologous strains were found in recent, and heterologous ones in older groups. Ogawa strain and three other strains which were heterologous to the Ogawa strain were tested with anti-Mahoney and anti-Brunenders sera, to test the specificity of the wild strain isolated in Japan. None of these showed similar antigenicity to the Mahoney or Brunenders strains. Moreover, they were more heterologous to these antisera than other laboratory strains (Figs. 1 and 2).

Absorption test of sera with active virus

Some antigenic differences were shown between wild strains by preliminary neutralization tests. However, the normalized K values of 2 in 3 slowly neutralizing strains were above 60. The serum absorption test was performed to confirm the small antigenic difference. Model absorption tests were done with anti-LSc 2ab serum and a few type 1 strains. Anti-LSc 2ab serum was diluted to 1 : 64

Table 3. Preliminary Test of Wild Type I Viruses with Anti-Ogawa Serum

Name	Isolated in		NK value
	Place	Year	
Ogawa	Osaka	1961	100
Yutani	Hokkaido	1956	62
Matsubara	Tokyo	1959	64
Hirasawa	〃	1959	43
Fukuda Y	〃	1960	98
Y-12-61	Yamaguchi	1961	98
Y-2-61	〃	1961	102
Ogu	Hokkaido	1958	103
Kawasaki	〃	1959	97
Yudono	〃	1960	109
Sotodate	〃	1960	117
Mahoney			34

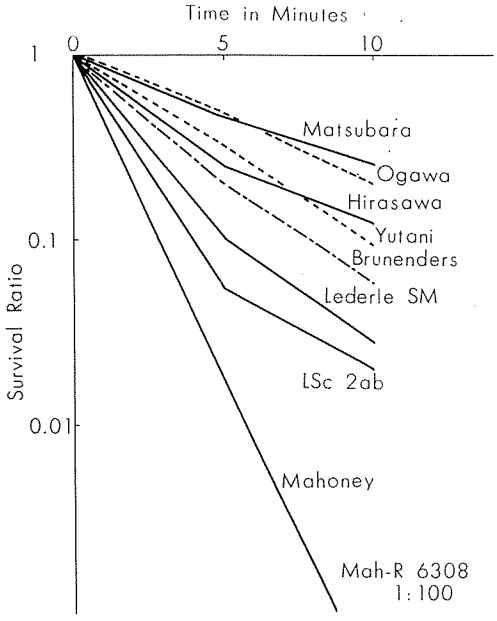

Fig. 1. Kinetic Neutralization of Wild Strains with Anti-Mahoney Serum

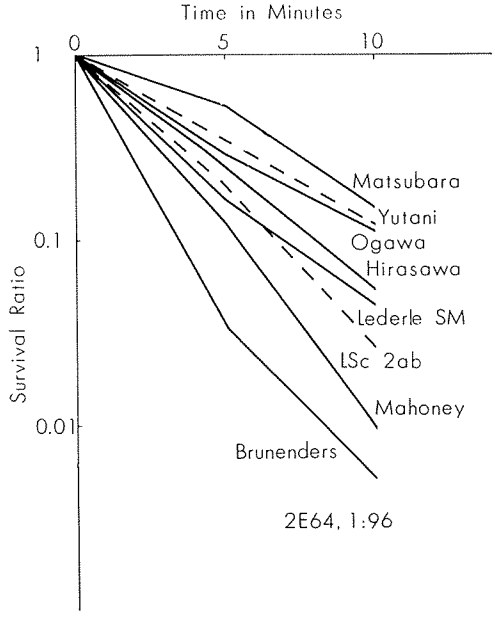

Fig. 2. Kinetic Neutralization of Wild Strains with Anti-Brunenders Serum.

with PBS and absorbed with homologous, Brunenders and Ogawa strains.

Control serum was mixed with Hanks' solution and treated in the same way. After heating at 56°C for 30 min., there was no detectable amount of virus in these treated sera. Kinetic neutralization tests were done with these sera. Fig. 3a shows the neutralization with control serum. As shown in Fig. 3b, homologous virus absorbed the neutralizing antibody sufficiently and no specificity of the serum could be demonstrated. On the other hand, absorption with the heterologous strain did not reduce the neutralizing capacity of the serum so much as homologous virus, but the capacity to neutralize the same strain as used for absorption was evidently reduced (Figs. 3c and 3d).

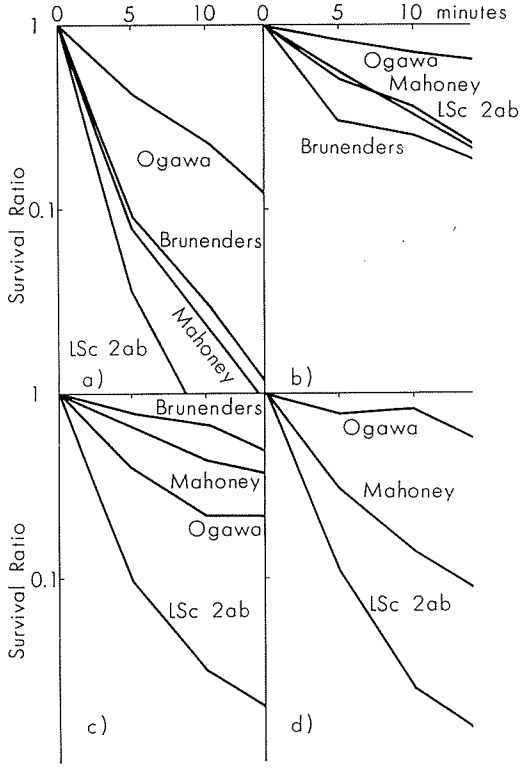

Fig. 3a-d. Absorption Test of Anti-LSc 2ab Serum with Three Immunologically Distinct Strains

a) Control
b) Absorption with LSc 2ab
c) Absorption with Brunenders
d) Absorption with Ogawa

The absorption test was applied on Anti-Ogawa serum with one homologous (Fukuoka Y) and one intermediate (Matsubara) strain. In both cases, the capa-

city to neutralize homologous virus was not greatly affected, but the Fukuoka Y strain showed a similar velocity of neutralization as to the Ogawa strain before and after absorption, though the NK value of the Matsubara strain was greatly reduced after absorption (Figs. 4a-c).

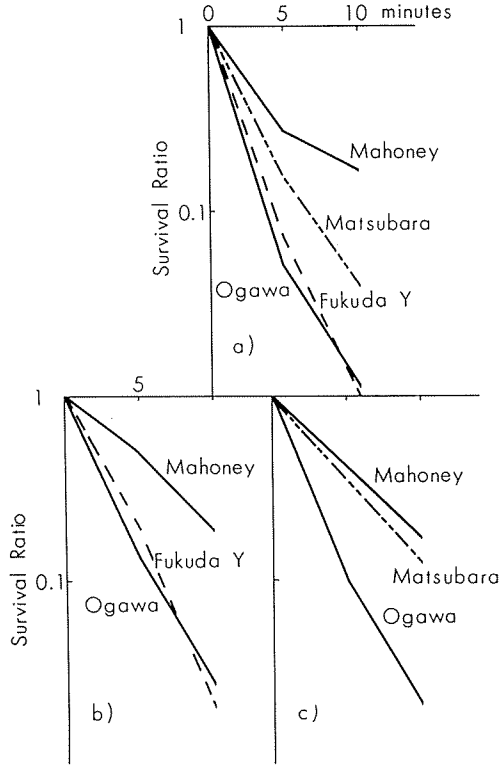

Fig. 4a-c. Absorption Test of Anti-Ogawa Serum with 2 Wild Strains
a) Control
b) Absorption with Fukuda Y
c) Absorption with Matsubara

Reciprocal neutralization of three immunologically similar strains with their anti-sera

Many strains isolated in different areas in different years were neutralized rapidly with anti-Ogawa serum. To confirm their antigenic similarity, two strains were selected from different areas as representative strains of the Ogawa homologous group. The Ogawa strain and these two strains were tested by reciprocal neutralization with their anti-sera. All the strains showed NK values of above 90 with each of the three anti-sera and no antigenic difference was observed between these strains. This result suggests the antigenic identity of the epidemic strains in the three different areas (Table 4).

Table 4. Reciprocal Neutralization Test of Three Representative Strains

Virus		NK value with Anti-serum		
Strain	Isolated in	Ogawa	Kawasaki	Y-12-61
Ogawa	Osaka 1961	100	96	100
Kawasaki	Hokkaido, 1959	97	100	113
Y-12-16	Yamaguchi, 1961	98	102	100
K value with isologous virus		69.1	689	27.0

Wild strains in various areas

One hundred and twelve strains were tested with anti-Ogawa serum and some of them, several Ogawa-homologous, Ogawa-similar and a few Ogawa-heterologous strains, were further tested with anti-Kawasaki and anti-Y-12-61 sera. Representative results of the tests are shown in Tables 5,6 and 7.

All strains isolated in the northern part of Japan are included in Table 5. The Yutani strain is somewhat different from the Ogawa strain and heterologous to the Kawasaki strain. Two other strains isolated in the same year are also different from the Ogawa strain. In 1958 Ogawa-homologous strains appeared in Hokkaido and one of them, the Ogu strain, is homologous to the Kawasaki and Y-12-61 strains. Since that year, no heterologous strain was observed in Hokkaido until mass vaccination with oral vaccine was performed. The Ishigaki strain is a little different antigenically from the Ogawa and Kawasaki strains, but considering the high specificity of anti-Kawasaki serum this strain may be included in the recent

Table 5. Screening Test of Wild Strains Isolated in Northern Part of Japan with Anti-sera against the Recent Epidemic Group

Strain	Isolated in		NK value with anti-serum		
			Ogawa	Kawasaki	Y-12-61
Yutani	Hokkaido,	1956	62	49	ND
Ogu	〃	1958	103	105	110
Kawasaki	〃	1959	97	100	113
Yudono	〃	1960	95	105	108
Ishigaki	〃	〃	89	87	ND
Shiraishi	Aomori,	1959	79	52	ND
Hakoishi	〃	〃	83	62	ND
Y-4	Yamagata,	1959	110	100	ND
Y-32	〃	〃	115	76	ND

ND : not determined

Table 6. Screening Test of Wild Strains Isolated in Kinki District with Anti-sera against the Recent Epidemic Group

Strain	Isolated in	NK value with anti-serum		
		Ogawa	Kawasaki	Y-12-61
Takashima	1956	93	58	
Arishima	1956	89	44	
Futakami	1957	106	74	
Korosue	1957	88	50	
Kihara	1957	102	72	
K-31	1958	101	98	
Kiyohara	1960	96	85	
Ogawa	1961	100	92	100
Tanino	1961	98	90	

Table 7. Screening Test of Wild Strains Isolated in Western Part of Japan with Anti-sera against the Recent Epidemic Group

Strain	Isolated in		NK value with anti-serum		
			Ogawa	Kawasaki	Y-12-61
Watanabe	Ehime,	1957	87	57	62
Yata	Yamaguchi,	1957	85	65	73
Y-12-61	Yamaguchi,	1961	98	102	100
K-151	Kyushu,	1958	85	91	
Matsushita	Kagoshima,	1959	104	97	84
A-12-60	Fukuoka,	1960	101	92	
M-34-60	〃	〃	107	108	
M-46-60	〃	〃	103	102	
F-40-61	〃	1961	102	110	
S-2-61	Saga	1961	100	105	
S-5-61	〃	〃	105	85	

epidemic group. In the Tohoku district only 2 Ogawa-homologous strains were found among the 10 tested. One of them is homologous to the Kawasaki strain, but the other, Y-32, showed intermediate characters in repeated tests. This difference in NK values may be interpreted as due to a difference in specificities of the anti-sera and the Y-32 strain may not be identical with the recent epidemic group.

Most of the wild strains in the Kinki district (central part of Japan) were obtained from Osaka, one of the most densely populated areas. As shown in Table 6, strains obtained in 1956 and 1957 are antigenically distinct from the

Kawasaki strain and these are considered to be heterologous to the recent epidemic group. A wild strain homologous to the recent epidemic group was first observed in 1958, but not in the following year. Both homologous and heterologous strains were observed in 1960 and 1961.

The most distinctive feature in the western part of Japan is that of the 8 strains tested only strains homologous with the recent epidemic group were observed in 1960 and 1961 (Table 7).

Tests on Ogawa-heterologous strains

Small scale field trials were done with Lederle live poliovirus vaccines in a few areas during 1958 to 1960. Four Ogawa-heterologous strains were tested by reciprocal neutralization with anti-Lederle SM and anti-Hirasawa (wild, Ogawa-heterologous) sera. The K-39 strain isolated in Tokyo in 1959 was added as a control strain. All these strains were distinct from the Lederle SM and Hirasawa strains (Table 8).

Table 8. Neutralization Test of Ogawa-heterologous Strains with Anti-Ogawa, Anti-Lederle SM and Anti-Hirasawa sera

Virus			Normalized K value with Anti-		
Strain	Isolated in		Ogawa	Lederle SM	Hirasawa
Ogawa	Osaka	1961	100	26	55
Lederle SM	Lederle Vaccine		ND	100	77
Hirasawa	Tokyo	1959	43	72	100
Y-5	Yamagata	1959	39	31	48
Nakao	Osaka	1958	44	45	66
Toyoda	Osaka	1960	50	51	70
K-39	Tokyo	1958	83	ND	50

ND: Not determined

Distribution of the recent epidemic strain

A screening test of the recent epidemic strain was done with anti-Ogawa serum and the results were corrected by a test with anti-Kawasaki serum. From the results of tests on many strains, the following criterion was adopted for classification of wild strains; a) strains showing a NK value of more than 90 with anti-Ogawa serum and of more than 85 with anti-Kawasaki serum are considered to be recent epidemic strains, b) those showing NK values between 80 and 89 with both anti-sera are also homologous, but it is uncertain whether they are recent strains, c) strains tested with anti-Ogawa serum alone and showing NK values of above 80 are questionable cases, d) those showing NK values of above 80 with anti-Ogawa serum, but below 79 with anti-Kawasaki serum are considered to be intermediate types, e) strains showing NK values of below 79 with anti-Ogawa and, or, anti-

Kawasaki are distinct from the recent epidemic strain. The results of the test were summarized in Fig. 5.

Fig. 5. Distribution of the Recent Epidemic Strain of Type 1 Poliovirus

Year	Northern part		Central part			Western part		
	Hokkaido	Tohoku	Kanto	Chubu	Kinki	Shikoku	Chugoku	Kyushu
1956	e e e				d d c e	e e		
1957					d d d e	d	d c e e e e	
1958	ⓐ ⓐ e		c e	e	ⓐ c e e e e e e		e e	c e e e e e
1959	ⓐ	ⓐ d e e e e e [d e]	e e e	e	c e e e e e e e e e e			ⓐ c e e
1960	ⓐⓐⓐⓐ ⓑ	[e e]	ⓐ ⓑ ⓑ c c c d e e		ⓐⓐⓐⓐ ⓐ c e e e			ⓐ ⓐ ⓐ
1961			ⓐ e		ⓐⓐⓐⓐ c e		ⓐ ⓐ	ⓐ ⓐ ⓐ

□ : Aomori prefecture
ⓐ : strains showing NK values of more than 90 with anti-Ogawa serum and of more than 85 with anti-Kawasaki serum
ⓑ : those showing NK values between 80 and 89 with both anti-sera
c : tested with anti-Ogawa serum alone and NK values above 80
d : those showing NK values above 80 with anti-Ogawa serum, but below 79 with anti-Kawasaki serum
e : strains showing NK values below 79 with both anti-sera
ⓐ and ⓑmay belong to the recent epidemic group

Fig. 6. Map showing Areas mentioned in Tables

Table 9. Number and Morbidity Rate of Poliomyelitis in Serveral Prefectures from 1956 through 1961

	Northern part		Central part			Western part		
	Hokkaido	Aomori	Tokyo	Aichi	Osaka	Ehime	Yamaguchi	Fukuoka
1956	203 4.2	124 8.8	57 0.7	25 0.7	124 2.6	139 9.0 I	37 2.3	125 3.2
1957	122 2.5	67 4.7	59 0.7	32 0.8	85 1.7	71 4.6	244 15.0 I	119 3.0
1958	207 4.2	42 2.9	86 1.0	50 1.2	129 2.5	31 2.0	179 11.0 II	321 I 8.0 II
1959	94 1.9	141 9.8 I	339 3.6	97 2.4	159 3.0	27 1.7	50 3.1	145 3.6
1960	1600 31.8 I	87 6.1	281 2.9	391 9.3	103 1.9	95 6.1	272 17.0 I	273 6.8 I
1961	67 1.3	10 0.7	223 2.2	44 1.0	119 2.1	13 0.9	84 5.3 I	300 7.5 I

Upper numbers represent the number of patients and lower numbers represent the morbidity rate (per 100,000).
Roman numerals indicate virus type isolated during epidemic stage.

From the above mentioned criterion, the recent epidemic strain appeared first in 1958 in Hokkaido and in Osaka (Kinki), but this strain was not dominant as wild type 1 poliovirus during 1958 and the following years. In 1960 a large scale epidemic occurred in Hokkaido and another epidemic occurred in the Chugoku-Kyushu district in 1960 and 1961 (Fig. 6 and Table 9). All strains isolated in these areas during these years are antigenically homologous with each other and these are considered to be the recent epidemic group. On the other hand, both in Tokyo (Kanto) and in Osaka (Kinki), no large epidemic occurred during these years (Table 9) and strains isolated in these areas during these years are a mixture of the recent epidemic strains and antigenically different strains. Aomori (Tohoku) is close to the south of Hokkaido and epidemics were observed in 1959 and in 1960. However, 4 strains isolated in this prefecture in these years are heterologous to the recent epidemic group observed in Hokkaido and in the Chugoku-Kyushu district (Figs. 5, 6 and Table 9).

DISCUSSION

In previous papers on the antigenic analysis of type 1 polioviruses, McBride (1959) noted that strains isolated from one area within a single year appeared to be more similar antigenically than strains isolated in other areas and at other times,

and Wenner *et al.* (1959) also noted that there exist in nature type 1 polioviruses sufficiently different immunologically from Brunhilde and Mahoney viruses. Thirteen type 1 polioviruses isolated in Japan in 1958 were also heterologous to the Brunhilde strain (Kono, 1960).

The present study indicates that wild type 1 polioviruses isolated in Japan also differ immunologically from laboratory strains and that various grades of immunological difference can be observed among these wild strains. To confirm whether these differences were caused by technical errors, the absorption test of serum was performed. Active viruses were employed for the absorption tests on sera, since some workers failed to demonstrate the validity of the absorption test on sera using inactivated virus (Gard, 1960; Plotkin *et al.*, 1961). A model experiment was done with anti-LSc 2ab serum and various strains of virus. The capacity to neutralize the virus was markedly reduced by absorption with the LSc 2ab strain and no specificity of the serum could be demonstrated. Absorption with heterologous strains affected the velocity of the neutralization of the virus employed for absorption, although K values of other viruses were not affected so much as by absorption with LSc 2ab strain. This suggests that a qualitative rather than quantitative difference is responsible for the intratypic difference in antigenicity. The absorption test was applied in serological differentiation of 2 wild strains with different NK values. The results suggest that even small differences in the NK values in neutralization kinetics is promising for discrimination of heterologous virus from the homologous group.

Three Ogawa-homologous strains were selected from 3 distinct areas of Japan, and these were tested by reciprocal neutralization by the kinetic method with their anti-sera. No immunological difference was observed between these strains and these are considered to be definitely the virus which caused epidemics in the recent years. Then a screening test of Ogawa-heterologous virus was done with anti-Ogawa serum and that of the recent epidemic group virus was done with anti-Kawasaki serum, since the former exhibited a broader range of neutralization than the latter (Tables 5, 6 and 7). From these tests it was shown that epidemics of poliomyelitis in Hokkaido in 1960 and in Chugoku-Kyushu from 1960 to 1961 (Table 8 and Fig. 6) are caused by an antigenically homologous group of type 1 virus and no, or very few, heterologous strains were present in these areas in these years, although these two areas are quite far apart geographically. On the other hand, a mixed population of type 1 strains was observed in Tokyo and in Osaka in the central part of Japan. Although no definite conclusion can be made on the source of the recent epidemic strains, those homologous with the recent epidemic group have been isolated since 1958 in Hokkaido and in Osaka, and in 1959 in Kyushu. On the contrary, strains heterologous to the recent epidemic group were isolated in Aomori in 1959 and 1960 during local epidemics, though Amoroi is adjacent to Hokkaido. Moreover, most type 1 strains isolated in 1959 in Yamagata (Tohoku, to the south of Aomori) are also heterologous to the group. These differences in

the mode of spread of this group between rural areas and large cities may be explained by differences in their ecology. The dense population of large cities, *i.e.* Tokyo and Osaka, permits type 1 wild strain to be prevalent constantly and this may affect the invasion of newer strains by interference or by inducing immunity among the inhabitants. In such areas, no large epidemic has been observed (Table 8). On the other hand, in rural areas including some small but densely populated towns (Hokkaido and Chugoku-Kyushu areas), the scarcity of preceding virus promotes an increase of susceptible populations and permits the invasion of new strains. When a virulent virus is introduced into such an area, large epidemics may occur. In fact the increase in a susceptible population was noted in Hokkaido before the epidemic in 1960 (Kanamitsu, 1960). The latter areas are almost filled up with a single strain in a relatively short period and other strains become scarcely detectable. Aomori may also belong to the latter group and may have been filled up with antigenically distinct strains before the invasion of the recent epidemic group.

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