PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 254–258 (1974)

Comparative Studies of the Viruses Rescued from Polyoma Tumor Cell Lines[1] (38081)

FUMIAKI TAGUCHI
(Introduced by I. Yamane)
Department of Microbiology, Kitasato University School of Hygienic Sciences, Sagamihara, Kanagawa-ken, 228 Japan

Polyma virus has many characters comparable to simian virus 40 (SV40) and convert normal cells to malignant cells. Although the cells transformed by oncogenic DNA viruses generally produce neither viral antigen nor infectious virus, the persistence of the viral genome in these transformed cells is demonstrated by the presence of new virus-induced specific antigens (1) and RNA complementary to viral DNA (2). The presence of viral DNA in the transformed cells was demonstrated by hybridization technique (3), and it was shown to be integrated into cellular DNA (4, 5).

Viral genome can be activated by cell fusion in certain SV40 transformed cells (6, 7), but activation of polyoma viral genome in the transformed cells has not been successful except in a few reports (8–10). With regard to SV40, the rescued viruses from the transformed cells have had different properties from each other. Takemoto *et al.* (11) isolated the virus which had an identical marker in plaque morphology with that of the parental virus. Todaro and Takemoto (12) obtained the virus which had a higher cell transformation ability than that of the parental virus.

Recently, we have succeeded in activation of the viral genome in polyoma tumor cells and have isolated two viruses (9). The present paper is concerned with comparative studies of some biological properties of those rescued viruses together with the parental virus.

[1] This work was supported in part by a Grant from the Ministry of Education of Japan.

Materials and Methods. Polyoma virus. A large plaque strain of polyoma virus (St-Lp) originally obtained from Dr. V. Defendi of the Wistar Institute was passaged in cultured mouse cells. R5/2 and R20 were the viruses rescued from the polyoma tumor cell lines, KIT-5/2 and KIT-20W, respectively, after the passage in mouse kidney cells (9).

Cells. Both primary culture of mouse kidney cells (MK) and secondary culture of mouse embryonic cells (ME) were prepared from CFW and dd mouse freed from polyoma infection. Established mouse cell line of Swiss 3T3 was kindly supplied through the courtesy of Dr. S. Uchida of National Institute of Health (Tokyo).

Medium. Eagle's minimal essential medium (MEM) containing 10% calf serum was used for the growth of ME and 3T3. MK cells were grown in a mixed medium of MEM and LH (Hanks' salt solution with 0.5% lactalbumin hydrolysate) supplemented with 10% calf serum and 10% tryptose–phosphate broth (Difco).

Infectivity titration. The 50% infectious dose for tissue culture cells ($TCID_{50}$) was determined by inoculating specimens into the tube cultures of MK or ME.

Plaque assay. The monolayer cultures of 3T3, MK, and ME were prepared in 60-mm Petri dishes. After adsorption of virus for 2 hr at 37°C, the infected monolayer was overlaid with 6 ml of an agar medium consisting of 2.5% horse serum, 0.9% agar, and 10% tryptose–phosphate broth in MEM. The second and third overlays in an amount of 4 ml each were made at 4-day

intervals. The third overlay contained 0.1% of neutral red. Plaques were measured 2 and 3 wk after the virus infection.

Preparation of immune sera. CFW mice with an average weight of 20 g were inoculated intraperitoneally with 0.2 ml of polyoma virus containing about 10^8 $TCID_{50}$/ml and killed 2 wk after the inoculation. Sera from 10 mice were pooled and used for HI test.

Hemagglutination-inhibition (HI) test. HI tests were performed by mixing 0.2 ml of two-fold dilutions of heat-inactivated antiserum with an equal volume of 8 HA units of virus suspension for 60 min at room temperature. The mixture was added with 0.4 ml of the guinea pig blood cell suspension and HI titers were read 4 hr after incubation at 4°C.

Transformation of hamster cells. Following the method described by MacPherson and Montagnier (13), primary hamster embryonic cell cultures were used for the transformation experiment. The cells in suspension were infected with polyoma virus at an input multiplicity of 100 $TCID_{50}$/cell at room temperature for 60 min. After low speed centrifugation, the cells were resuspended into the soft agar to make 10^5 cells per plate and placed on the top of the solid agar. The incubation was continued for 2–3 wk at 37°C and the resulting colonies of the transformants were counted under microscope.

Results. Isolation of plaque size mutants of polyoma virus. Plaque assay of the parent (St) and the rescued viruses (R5/2 and R20) was carried out on the monolayer cultures of ME. Plaque size was first scored 2 wk after virus infection. The St strain produced only large plaques with diameter of 4–6 mm (St-Lp). Two types of plaques were produced by R5/2, the small one (R5/2-Sp) with diameter of less than 1 mm and the large one (R5/2-Lp) having about 2 mm diameter. More than 95% of the plaques consisted of R5/2-Sp. On the other hand, plaque morphology produced by R20 was only of a small type with diameter of less than 1 mm (R20-Sp). These plaque size differences were also observed on 3T3 and MK cultures. Development and distribution of plaque size are summarized in Table I.

Antigenic relationship of the rescued viruses. Antigenic relationship among the parent and the rescued viruses were studied by HI test. Table II shows the antigenic differences between the large and the small plaque viruses. The antisera prepared against each of the large plaque viruses, e.g., St-Lp and R5/2-Lp reacted almost equally with both of the large plaque viruses. They also reacted with the small plaque viruses, the HI titer being one-half to one-fourth of that obtained in the homologous system. In the case of the antisera prepared against the small plaque viruses, they reacted equally with both of the small plaque viruses but did poorly with the large plaque viruses. Results from repeated experiments with different serum pools and also with serum pools harvested from mice at 3 mo after each virus inoculation showed almost the same antigenic relationship as presented in Table II, although little variations in the HI titers were obtained with different sera. These results seem to have disclosed the difference in the antigenic structure between

TABLE I. Plaque Formation by the Rescued Polyoma Viruses.

Viruses	Source	Plaque size (mm) [a] 2 wk incubation	Plaque size (mm) [a] 3 wk incubation	Proportion (%)
St-Lp	St	4–6	5–8	100
R5/2-Lp	R5/2	2	3–5	<5
R5/2-Sp	R5/2	<1	<1–2	>95
R20-Sp	R20	<1	<1–2	100

[a] Average of three determinations.

TABLE II. Antigenic Relationship of the Parental and the Rescued Polyoma Viruses.

	HI titers			
	Antiserum prepared against			
Antigens	St-Lp	R5/2-Lp	R5/2-Sp	R20-Sp
St-Lp	3200[a]	3200	400	400
R5/2-Lp	6400	3200	400	800
R5/2-Sp	1690	800	3200	3200
R20-Sp	1600	800	3200	3200

[a] Reciprocal of titer.

the large plaque and the small plaque viruses. Hare and Chan (14) have previously reported similar results of polyoma virus antigenic reactivity between large- and small-plaque strains. However, the antigenic difference between the small plaque viruses have not yet been detected.

Tumor induction in mice. One-day-old newborn mice of CFW strain and of dd strain were inoculated subcutaneously on the back with 0.05 ml of R5/2-Sp, R5/2-Lp, and St-Lp (10^8 $TCID_{50}$/ml). The mice were examined for tumor formation by weekly palpation and all surviving mice except ones dying with tumor or by accidents were killed for pathological examination 6 mo after the inoculation. More than half of the mice inoculated with St-Lp developed tumors (Table III). The rate of the tumor induction was not significantly different in the two strains of the mice used. However, in case of the rescued viruses, few mice developed tumors; R5/2-Lp induced tumors in 2 out of 13 dd mice and R5/2-Sp, in none out of 9 CFW mice, and in 1 out of 30 dd mice. Low tumorigenicity of R5/2-Sp was ascertained by inoculating the mice with 100 times the amount of the virus (10^{10} $TCID_{50}$/ml). It was found that none of 14 dd inoculated mice produced tumor. It should be noted that the tumorigenicity of the rescued large plaque virus, R5/2-Lp, was significantly lower (Fisher's method, $p = 0.025$) than that of the parental large virus, St-Lp. None of the enhancement in the tumorigenicity was observed by a mixed infection of R5/2-Lp and R5/2-Sp.

In vitro transformation by the rescued viruses. Hamster embryonic cells in suspension were infected with the parental and the rescued viruses at an input multiplicity of 100 $TCID_{50}$/cell. It is evident from Table IV that the rescued viruses of R5/2-Lp and R5/2-Sp transformed the cells far less frequently than the parental virus of St–Lp did. The results, together with those of tumor induction *in vivo,* show that the cell transforming and also tumorigenic abilities of both rescued viruses, R5/2-Lp and R5/2-Sp, were significantly reduced in comparison with those of the parental virus, St-Lp, and especially among them, the small plaque virus, R5/2-Sp was the least active for the alteration of cells *in vivo* and *in vitro.* The mouse cells transformed by the rescued viruses were demonstrated to produce the polyoma specific T antigen (unpublished data), but attempts to rescue the viral genome from these cells have failed.

TABLE III. Tumor Induction by the Rescued Viruses.

Viruses	Virus titer per ml	Mouse strains	
		CFW	dd
St-Lp	10^8 $TCID_{50}$	4/8[a]	9/15
R5/2-Lp	10^8 $TCID_{50}$	NT[b]	2/13
R5/2-Sp	10^8 $TCID_{50}$	0/9	1/30
R5/2-Sp	10^{10} $TCID_{50}$	NT	0/14
R5/2-Lp plus R5/2-Sp[c]	$10^{8.3}$ $TCID_{50}$	2/14	NT

[a] No. of mice with tumors/no. of mice examined.

[b] Not tested.

[c] R5/2-Lp and R5/2-Sp at $10^{8.3}$ $TCID_{50}$/ml were mixed in equal proportions and 0.05 ml of the mixture was inoculated into each mouse.

TABLE IV. Hamster Cell Transformation by the Rescued Viruses.

Viruses	No. of colonies per plate	
	Expt I	II
St-Lp	59[a]	86
R5/2-Lp	24	41
R5/2-Sp	6	12

[a] Values are means of 10 plates per virus in each of which a total of 10^5 infected cells were plated.

Discussion. Comparative studies of the viruses rescued from two polyoma tumor cell lines were carried out by biological procedures. The plaque size mutants were isolated from the rescued virus progenies. Significant differences were found between the large plaque and the small plaque viruses in the antigenic reactivity. Regardless of plaque size, the rescued viruses were less oncogenic in mice and also induced transformation *in vitro* less frequently than the parental virus. No difference could be found between the small plaque viruses by the test so far made.

The mouse cells transformed by the rescued viruses as well as the parental virus were demonstrated to produce the polyoma specific T antigen (unpublished data), but attempts to rescue the viral genome from these cells have failed so far. It has been observed widely that the small plaque lines derived from polyoma virus were less oncogenic than the large plaque lines derived from the same virus (15–17). On the other hand, it has been reported that a small plaque mutant isolated from L cells persistently infected with polyoma virus had higher transforming ability than the parental large plaque virus (18). The present experiment showed that there was no direct correlation between the oncogenicity *in vivo* or *in vitro* and the plaque size of polyoma virus used.

The antigenic difference between the large plaque and the small plaque viruses was shown by HI test and this may be a reflection of the modification of the rescued viral DNA, although the difference between the large plaque rescued virus and the parental virus could not be disclosed by this test.

Through the present study, it may be worthwhile to note that the same plaque viruses rescued independently from the different cell lines had properties indistinguishable from each other. This may suggest either a nonrandom rescue of the polyoma virus genome from the cells or a regularity in the process of the integration of the viral DNA into the cellular DNA, although the genetic stability of the plaque size revealed by the parent virus should be exhaustively studied. More intensive studies on rescued viruses may give better understanding of these problems and of polyoma virus oncogenesis.

Summary. Biological studies were made on the viruses rescued from two polyoma tumor cell lines induced by the parental large plaque virus, St-Lp. The virus rescued from one of the cell lines, KIT-5/2, consisted of two different plaque formations, a large plaque, R5/2-Lp and a small plaque, R5/2-Sp, and the one rescued from the other cell line, KIT-20W, entirely of small plaques, R20-Sp.

The antigenic differences between the small plaque and the large plaque viruses were disclosed by HI test. Regardless of plaque size, all of the rescued viruses were shown to be less tumorigenic while the parental virus was highly tumorigenic. This situation held true also in the *in vitro* transformation study.

The excellent technical assistance of Y. Yoshida and J. Kajioka is greatly acknowledged. I thank D. Nagaki for the encouragement and support, and I. Yamane, H. Shimojo and M. Homma for valuable discussions.

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Received Sept. 4, 1973. P.S.E.B.M., 1974, Vol. 146.