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STUDIES ON LIVE MUMPS VIRUS VACCINE. V. DEVELOPMENT OF A NEW MUMPS VACCINE "AM 9" BY PLAQUE CLONING

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SUMMARY To obtain an improved vaccine, plaque cloning of mumps virus was done in quail embryonic fibroblast cell (QEF) cultures. Among 6 selected clones, clone U-1005-9 of the newly isolated mumps virus, Urabe strain, was chosen as seed virus of vaccine preparation after clinical tests in healthy children. This clone formed small plaques on chick embryonic fibroblast cells (CEF) and showed higher infectivity titers in human embryonic kidney cells (HEK) than in CEF cultures.

The effect of the passage level of the clone in the amniotic cavity of chick embryos (AM) and QEF on its immunogenicity in children was also tested. Its immunogenicity was high after up to 20 passages in Am but the antibody response to the clone decreased after more than 16 passages in QEF.

The minimal effective dose of virus to induce neutralizing antibody was $5\times10^{2.0}$ $TCID_{50}$. None of the vaccines used in clinical tests caused clinical manifestations in children.

INTRODUCTION

Previously we reported (Yamanishi et al., 1970b) results of field trials on live attenuated mumps virus vaccine prepared from the Urabe strain after serial passage in AM. No children injected with the vaccine showed any clinical reactions and children receiving vaccines prepared from the Urabe strain at the 4th, 6th, 9th, or 15th passage level showed an antibody response.

However, the seroconversion rates of children receiving the Urabe strain at the 21st and 26th passage levels were only 86.0% and 75.0%, respectively (Yamanishi, 1971). These results suggested that the Urabe strain gradually lost immunogenicity during serial passage in AM. To select clones having stable immunogenicity, irrespective of their passage level in AM, plaque cloning of the Urabe strain at the 6th

passage level in AM (U-1005) was done in quail embryonic fibroblast cells (QEF).

This report describes some biological characteristics of the selected clones and results of clinical tests on the vaccines prepared from these clones.

MATERIALS AND METHODS

1. *Virus cloning and preparation of vaccine*

Virus of the Urabe strain passaged 6 times in AM (U-1005) was inoculated onto QEF in plastic dishes of 60 mm diameter (Toyoshima works, Tokyo) and incubated for two hr in an atmosphere of 5% CO_2 in air (CO_2-incubator) at 37 C. After incubation the cells were washed once with phosphate buffered saline and overlayed with 6 ml of medium consisting of Medium 199, 0.36% skimmed milk (Difco Lab.) 0.05% pancreatin (Nutritional Biochemicals Co.), 0.15% $NaHCO_3$ and 0.9% agarose. Infected QEF cells were kept at 37 C in a CO_2-incubator and overlayed with 3 ml of the medium every four days. The cells were stained by adding 3 ml of medium consisting 0.01% neutral red on the 8th day after infection. Plaques of different sizes were recognized on the following day and 6 clones of different plaque sizes were selected. Plaque cloning was carried out twice. The selected clones were each passaged several times in AM or QEF.

The method for preparation of vaccine from the amniotic fluid was as described previously (Yamanishi et al., 1970b), while the method for its preparation from QEF was follows. Culture fluid free from serum was harvested on the 5th day after infection and clarified by centrifugation at 3,000 rpm for 30 min. The supernatant fluid was used as test vaccine.

2. *Plaque formation on chick embryonic fibroblast cells (CEF)*

The method of plaque formation on CEF was the same as that on QEF. The plaque size was measured on the 10th day after infection. In this paper large plaques are those of more than 4.0 mm diam and small plaques are those of less than 2.0 mm diam.

3. *Virus titration*

Infectivity of virus was titrated in HEK and CEF by the hemadsorption method with chicken red blood cells. The infectivity titer was read 7 days after infection at 37 C.

4. *Vaccination*

Vaccination was carried out during 1971–1973 in Suita City, Osaka, in healthy children living at home with a negative history of mumps.

A dose of 0.5 ml of vaccine was injected subcutaneously. Blood specimens were collected just before and four to six weeks after vaccination. Serum specimens were inactivated by heating at 56 C for 30 min before the neutralizing test.

5. *Neutralizing (NT) test*

Neutralizing tests were done in microplates instead of tubes. Microplates (Linbro Co., New Haven, Conn., U.S.A.) were sterilized by UV-irradiation for 15 min. Then 0.025 ml of diluent (phosphate buffered saline containing of 0.2% of gelatin and 200 μg/ml Kanamycin) was dropped into each well with a 0.025 ml dropper (Microtiter, Cook Engineering Co., Alexandria, Va., U.S.A.). Serum specimens were serially diluted in this well with 0.025 ml-diluters which had been sterilized in a flame before use. Then 0.025 ml of antigen, containing about 100 $TCID_{50}$ of mumps virus (Miyake strain) was dropped into each well and the serum-antigen mixture was shaken well. After incubation for one hour at 37 C, the mixture was kept overnight at 4 C. On the following day 0.1 ml of LLC-MK_2 cells (about $2\times10^{4.0}$ cells/0.1 ml) was added to the serum-antigen mixture. The microplate was incubated at 37 C in the CO_2-incubator. The culture fluid was decanted on the 6th or 7th day after inoculation and 0.05 ml of a 0.5% suspension of chicken red blood cells was put into each well. The microplates were kept at room temp for half an hr. The neutralizing antibody titers were measured by the hemadsorption pattern which was similar to that in the HI test.

RESULTS

1. *Biological characteristics of six clones*

Titration for virus infectivity was carried out in HEK and CEF and plaque assays were done in CEF, using each clone which had been passaged once in AM after cloning.

As shown in Table 1, the ratios of infectivity titers obtained in HEK and CEF varied from 1: 10 to 1: 1. On the other hand, that of the T-All-16 strain, which is known as a over-

TABLE 1. *CEF/HEK titer ratio and plaque size on CEF cells of six clones and T-A11-16 strain*

Virus clone	Virus titer in HEK cells log/0.1 ml	Virus titer in CEF cells log/0.1 ml	CEF/HEK ratio	Plaque size on CEF cells
U-1005-1 AM 1[a]	6.5	5.5	1/10	small
U-1005-2 AM 1	6.5	6.5	1/1	large
U-1005-3 AM 1	6.5	6.5	1/1	large
U-1005-9 AM 1	6.5	6.0	1/3.3	small
U-1005-16 AM 1	6.5	6.0	1/3.3	small
U-1005-18 AM 1	6.5	6.5	1/1	large
T-All-16[b]	3.5	6.0	300/1	large

[a] Urabe strain, clone 1, purified by plaque isolation twice in QEF and passaged once in AM.
[b] Towata strain, passaged 16 times in the chorioallantoic cavity of chick embryos.

TABLE 2. *Antibody responses to six clones*

Virus clone	No. of vaccinees	Virus titer $TCID_{50}$/dose	NT antibody response	
			Seroconv. rate (%)	G. M. titer[a]
U-1005-1 AM 1	87	$5\times10^{4.0}$	38/39 (97.4)	1:8.1
U-1005-2 AM 1	53	$5\times10^{4.0}$	23/24 (95.8)	1:9.9
U-1005-3 AM 1	81	$5\times10^{5.0}$	28/38 (73.7)	1:7.2
U-1005-9 AM 1	125	$1\times10^{5.0}$	45/46 (97.8)	1:6.3
U-1005-16 AM 1	43	$5\times10^{4.0}$	23/26 (88.5)	1:3.5
U-1005-18 AM 1	37	$5\times10^{4.0}$	18/24 (75.0)	1:5.0

[a] Geometric mean titer.

attenuated strain from field tests (Okuno, 1969) was 300: 1.

The plaque sizes of clones, U-1005-1, U-1005-9, and U-1005-16 were small. On the other hand, those of clones, U-1005-2, U-1005-3 and U-1005-18 were large. Clones forming small plaques on CEF showed higher infectivity titers in HEK than in CEF. On the contrary, all clones forming large plaques on CEF, except U-1005-2, showed lower infectivity titers in HEK than in CEF.

2. *Serological responses of children to the six clones*

The six test vaccines prepared from selected clones of the Urabe strain were tested clinically in healthy children. No clinical manifestations were observed for 4 to 6 weeks after vaccination. As shown in Table 2, the seroconversion rates with the vaccines prepared from clones U-1005-1, U-1005-2, and U-1005-9 were 97.4%, 95.8% and 97.8%, respectively, while those with clones, U-1005-3, and 1005-18 were 73.0% and 75.0%, respectively.

The seroconversion rates were low with vaccines prepared from all cloes with large plaques of CEF except clone U-1005-2. On the contrary, those with clones forming small plaques on CEF were high. However, the antibody titers elicited by the six clones were similar.

3. *Passage levels in AM or QEF and minimal effective dose of clone U-1005-9 vaccine*

Of the six clones clone U-1005-9 was chosen as the seed strain of the new mumps virus vaccine. It was then serially passaged in AM. Vaccines prepared at the 1st, 5th, 10th, 15th,

and 20th passage levels in AM were tested clinically on healthy children.

These vaccines caused no clinical manifestations and all elicited good antibody responses (Table 3).

To find the minimal effective dose, 0.5 ml of serially diluted vaccine U-1005-9 AM 1 (1st passage in AM) and U-1005-9 AM 5 (5th passage in AM) were injected in children. Groups of children were given doses of $1\times10^{6.0}$, $5\times10^{5.0}$, $1\times10^{5.0}$, $5\times10^{4.0}$, $1\times10^{4.0}$, $5\times10^{3.0}$, $1\times10^{3.0}$, $5\times10^{2.0}$ and $1\times10^{2.0}$ TCD_{50} of vaccine U-1005-9 AM 1. Their serocoversion rates varied 100% to 71.4%. Only 10 of 14 children (71.4%) receiving a dose of $1\times10^{2.0}$ $TCID_{50}$ showed an NT antibody response. Thus a dose of more than $5\times10^{2.0}$ $TCID_{50}$ of living virus is necessary

TABLE 3. *Antibody response to clone U-1005-9 passaged in the amniotic cavity of chick embryos*

Vaccine virus	No. of vaccinees	Virus titer $TCID_{50}$/dose	NT antibody response	
			Seroconv. rate (%)	G. M. titer[b]
U-1005-9 AM 1[a]	65	$1\times10^{6.0}$	31/31 (100)	1:12.6
	66	$5\times10^{5.0}$	15/15 (100)	1: 6.6
	146	$1\times10^{5.0}$	52/55 (94.5)	1: 6.3
	202	$5\times10^{4.0}$	98/99 (99.0)	1:10.6
	77	$1\times10^{4.0}$	34/36 (94.4)	1:13.4
	38	$5\times10^{3.0}$	16/17 (94.1)	1:13.0
	160	$1\times10^{3.0}$	64/66 (97.0)	1:10.1
	52	$5\times10^{2.0}$	11/11 (100)	1: 7.1
	30	$1\times10^{2.0}$	10/14 (71.4)	1: 8.0
U-1005-9 AM 5	131	$1\times10^{6.0}$	40/40 (100)	1:10.5
	144	$1\times10^{5.0}$	68/68 (100)	1: 9.6
	82	$1\times10^{4.0}$	27/28 (96.4)	1: 9.1
U-1005-9 AM 10	42	$5\times10^{5.0}$	20/20 (100)	1: 8.6
U-1005-9 AM 15	18	$1\times10^{6.0}$	2/2 (100)	1: 4.0
U-1005-9 AM 20	42	$1\times10^{6.0}$	11/12 (91.7)	1:10.8

[a] Urabe strain, clone 9, purified by plaque isolation twice in QEF and passaged once in AM.
[b] Geometric mean titer.

TABLE 4. *Antibody response to clone U-1005-9 passaged in quail embryonic fibroblast cells*

Vaccine virus	No. of vaccinees	Virus titer $TCID_{50}$/dose	NT antibody response	
			Seroconv. rate (%)	G. M. titer[b]
U-1005-9 QEF 1[a]	74	$5\times10^{5.0}$	18/18 (100)	1:11.3
U-1005-9 QEF 2	44	$1\times10^{6.0}$	20/20 (100)	1: 5.9
U-1005-9 QEF 11	66	$5\times10^{5.0}$	28/29 (96.6)	1: 7.8
U-1005-9 QEF 16	14	$1\times10^{5.0}$	8/11 (72.4)	1: 3.7
U-1005-9 QEF 20	42	$5\times10^{5.0}$	8/12 (66.7)	1: 6.7

[a] Urabe strain, clone 9, purified by plaque isolation twice in QEF and passaged once in QEF.
[b] Geometric mean titer.

for a sufficient antibody response. Children injected with doses of $1\times10^{6.0}$, $1\times10^{5.0}$ or $1\times10^{4.0}$ $TCID_{50}$ of U-1005-9 AM 5 showed good NT antibody responses.

As shown in Table 4, clone U-1005-9 was also serially passaged in QEF and vaccines were prepared at the 1st, 2nd, 11th, 16th and 20th passage levels. The NT antibody responses with vaccines at the 1st to 11th passage levels were good. However, the seroconversion rates with vaccines prepared from the strain after 16 and 20 passages in QEF were only 72.7% and 66.7%, respectively, even though vaccines were injected with a dose of more than $1\times10^{5.0}$ $TCID_{50}$.

Vaccine viruses prepared at different passage levels in AM showed the same plaque sizes as that of U-1005-9 AM 1. However, the vaccine virus prepared at the 20th passage in QEF formed large plaques in CEF.

DISCUSSION

Hosai et al. (1970) reported that the Towata strain of mumps virus became attenuated when it was passaged in the chorioallantoic cavity of chick embryos and then in AM. In field trials with this mumps virus vaccine using the inhalation method the seroconversion rate was approximately 80% even with a large dose of virus, while when this vaccine was injected, the seroconversion rate was only 50% (Okuno, 1969). So it seemed that this mumps virus vaccine was over-attenuated.

To develop a live attenuated mump vaccine with good immunogenicity, a newly isolated mumps virus, the Urabe strain, was cultivated in AM. Using vaccines prepared from the strain at the 4th, 6th, 9th and 15th passage levels, no clinical manifestations were observed and the antibody response was satisfactory (Yamanishi et al., 1970b). However, after further passage of this strain in AM, vaccines prepared at the 21st and 26th passage levels gave seroconversion rates in children of only 86.0% and 75.0%, respectively (Yamanishi, 1971). Buynak et al. (1968) reported that their level A vaccine of the Jeryl Lynn strain (12th passage in CEF) was more immunogenic than level B vaccine (17th passage in CEF) but that the former induced swelling of the parotid glands in some vaccinees. When the latter was further passaged 10 times in CEF, its immunogenicity became lower (Buynak and Hilleman, 1966; Buynak et al., 1968).

As described above, mumps virus seems to be easily attenuated by serial passages in AM and becomes over-attenuated in the chorioallantoic cavity of chick embyos or CEF.

Plaque cloning of the Urabe strain (U-1005) in QEF was done in attempt to select a suitable strain as an appropriately attenuated virus, which did not lose immunogenicity during successive passages. It was found that our former mumps virus vaccine contained several variants which produced large and small plaques in CEF. As shown in Table 3, the seroconversion rates of children given clones forming large plaques were lower than those given clones forming small ones, except in the case of clone U-1005-2. The over-attenuated strain (T-A11-16) formed large plaques on CEF (Table 2), so large plaques probably increased during successive passages in AM.

The antibody responses of children to vaccines prepared from clone U-1005-9 at the 1st, 5th, 10th, 15th and 20th passage levels in AM and at the 1st, 2nd, 11th, 16th and 20th passage levels in QEF were compared. The antibody responses with AM-passaged vaccines were satisfactory. However, when the clone was further passaged more than 16 times in QEF, the seroconversion rate gradually decreased (Table 4).

We reported that the wild strain showed a higher titer in HEK than in CEF. On the other hand, the Towata strain, which has been passaged many times in the chorioallantoic cavity of chick embryos and shown to be an over-attenuated vaccine in clinical tests, gave a low titer in HEK (Hosai et al., 1970; Yamanishi et al., 1970a) and formed large plaques (Table 1). As shown in Table 1, when titrated in HEK and CEF, the six clones were inter-

mediate between the wild and over-attenuated strain.

When clone U-1005-9 was passaged up to 20 times in AM, its plaque size was small and stable. But when it was passaged up to 20 times in QEF, its plaque size became large.

So, from the results of clinical tests and of the biological characteristics of the strain, passage in the amniotic cavity seems more suitable than passage in QEF for preparation of live attenuated mumps virus vaccine. But the QEF passaged virus at between the 1st and 11th passages can also be used for vaccine.

Clone U-1005-2 formed large plaques on CEF and its infectivity ratio in HEK and CEF was 1: 1. However, it induced a good antibody response (Table 1, 2). If the antibody response of this clone is stable, a good vaccine can be prepared from it. Clinical tests on this clone are being carried out and results will be reported elsewhere.

In these studies, we selected clone U-1005-9 for preparation of vaccine and the antibody responses following vaccination became stable after plaque cloning. This vaccine was name "AM 9", since it was obtained by passage of clone 9 in the amniotic cavity.

As Weibel et al. (1967) reported, the titers elicited on vaccination are not so high as those elicited on natural infection. So the protective efficacy by mumps vaccine should be established by follow up studies.

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