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DEVELOPMENT OF A LIVE ATTENUATED VARICELLA VACCINE

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SUMMARY The Oka strain of varicella virus, isolated in our labolatory, was serially cultivated in guinea-pig embryo cultures (GPEC), and a considerable amount of cell-free virus was obtained from infected cells. GPEC passaged virus at the 6th passage level was used in a small scale field trial. Susceptible children of 1 to 10 years old were injected subcutaneously with 100 to 1,000 PFU of virus. No clinical reactions due to the vaccination were observed in any children, and a high rate of antibody response was obtained with viral doses of more than 200 PFU. Attenuated virus obtained by passage in GPEC was propagated in human diploid (WI-38) cells, and it was also effective in inducing an immune response without clinical reactions.

The results show that the Oka strain of varicella virus passaged in GPEC and human diploid (WI-38) cells may be used safely and effectively as a live attenuated vaccine.

INTRODUCTION

Varicella is a common and highly contagious disease characterized by fever and generalized vesicular exanthema. It is occasionally severe and even fatal in cases with chronic diseases, such as nephrosis or leukemia. Thus development and application of an effective live attenuated vaccine seems of value. We attempted to cultivate varicella virus serially in guinea-pig embryo cells (GPEC), and in this way we obtained a considerable amount of cell-free virus. This virus was then used for a small scale field trial on susceptible children, and a high rate of antibody response was obtained without clinical reactions. The attenuated virus was then propagated in human diploid (WI-38) cells, and found to be similarly effective in inducing antibody responses without clinical reactions in susceptible children. The present paper reports details of this work.

MATERIALS AND METHODS

1. *Virus*

The Oka strain of varicella virus was isolated from a 3 years old boy who was diagnosed clinically as having varicella. The virus was isolated in human embryonic lung (HEL) cells and identified as varicella virus by its typical cytopathogenicity in HEL cells (Fig. 1) and by serological tests, including fluorescent antibody staining (Fig. 2), and neutralization and complement fixation tests against sera of acute and convalescent cases (Table 1).

2. *Guinea-pig embryo cultures (GPEC) and human diploid cultures*

GPEC were obtained by trypsinization of skin-muscle tissues from 3–4 week old embryos. The cells were grown in 500 ml Roux bottles in a mixture of equal volumes of 199 medium and Eagle's MEM containing 10% calf serum. After inoculation of virus, maintenance medium containing 3% calf serum was used.

WI-38 (human diploid) cells were obtained from Flow Laboratories through Dainippon Pharm. Co., and used at the 27th to 32th passage level. The culture media were the same as those discribed above except that fetal calf serum was used instead of calf serum.

3. *Preparation of experimental vaccine and determination of viral infectivity*

Cell-free virus was obtained essentially as described by Caunt and Taylor-Robinson (1964) and Brunell (1967). When more than 70% of a monolayer in a 500 ml bottle showed cytopathic effects (CPE), the culture fluid was decanted and infected cells were washed and collected after EDTA treatment. Cells were suspended in 3 ml of Eagle's MEM and disrupted by ultrasonic treatment in a 20 kc sonifier for 30 to 40 sec. The preparation was centrifuged for 15 min at 4,000 rpm and the supernatant was used as experimental vaccine after addition of a suitable stabilizer. It was tested to ensure it was free from bacteria and mycoplasma and the absence of viruses other than varicella virus was confirmed by electronmicroscopy.

Infectious virus was measured as the $TCID_{50}$ using HEL cells or plaque assay in HEL cells. Plaque assays were done by inoculating virus onto HEL cell monolayers in 60 mm plastic dishes. After adsorption for 1 hour at 37 C, the first overlay medium (0.7% agarose in 199 medium containing 10% calf serum) was added. The second overlay was done on the 5th day and the final overlay with 0.1% neutral red was on the 10th or 11th day.

4. *Neurovirulence test of the vaccine virus*

To examine neurovirulence, 0.1 ml volumes of the undiluted vaccine virus were injected intracerebrally into 8 newborn guinea-pigs (within 48 hr of birth). Similarly, 3 cynomolgus monkeys were injected intrathalamically with 1 ml of undiluted vaccine virus. Injected animals were observed for 3 weeks, and then sacrificed for histological examination. After confirmation that the virus had no clinical or histological effects, the vaccines were used for field trials.

5. *Complement fixation test*

Antigen was prepared by the procedure of Schmidt et al. (1964). The Kawaguchi strain of varicella virus (isolated in our laboratory) was generally used, as this strain gave antigen of higher potency than other strains tested. When extensive CPE appeared in HEL cells in 500 ml bottle, infected cells were collected, suspended in 3 ml of phosphate buffered saline (PBS), sonicated for 3 min and centrifuged for 15 min at 3,000 rpm. The supernatant usually has an antigen titer of 1 : 32 or 1 : 64, and 4 units were used for the reaction. The microtiter method of Sever (1962) was used.

6. *Neutralization test*

The neutralization test was carried out essentially by the method of Caunt and Shaw (1969). The virus preparation was diluted and mixed with equal amounts of serial dilutions of serum or with Eagle's MEM as a control. After incubation at 37 C for 1 hr, with occasional shaking, 0.1 ml portions of the mixture were inoculated into each of three tubes of cells. The tubes were left at 37 C for 2 hr for adsorption of unneutralized virus, and then the medium was added. The tubes were incubated in stationary racks at 37 C for 5–6 days until viral lesions were large enough to count. No secondary foci developed within this time. The foci were counted using a low-power microscope.

7. *Field trials*

With consent from their parents, susceptible children were given various doses of virus in a volume of 0.5 ml. Parents were requested to check the body temperature and other clinical symptoms daily for 21 days after the injection. Serum was collected before and 1 month after vaccination and tested by the CF reaction.

8. *Fluorescent antibody staining*

The indirect method was used. After incubation with human varicella convalescent serum, anti-human-serum fluorescent rabbit serum was employed for staining.

TABLE 1. *Serological tests on varicella virus of Oka strain*

A) Neutralization test of Oka strain virus (HEL 7th passage)

	Number of plaques per tube with serum dilution						
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
With convalescent serum (14 days[a])	0, 0, 0	0, 0, 0	0, 0, 0	3, 2, 4	3, 3, 5	18, 21, 23	21, 27, 30
With acute serum (1 day)	27, 21, 32	26, 32, 35	21, 17, 28				
With Eagle's medium	18, 20, 17						

[a] Duration of rash when serum was taken.

B) Complement fixation test of Oka strain virus (HEL 7th passage)

Serum	Complement fixing antibody titer	
	Oka strain virus	Herpes simplex virus[b]
Acute serum (1 day[a])	<4	<4
Convalescent (16 days)	160	<4
Acute serum (1 day)	<4	<4
Convalescent (14 days)	320	<4

[a] Duration of rash when serum was taken.
[b] Herpes simplex virus type 1 (Miyama strain) kindly given by Dr. S. Nii.

TABLE 2. *Neutralization of GPEC passaged varicella virus (Oka strain, HEL 11th passage, GPEC 6th passage)*

	Number of plaques per tube with serum dilution						
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
With convalescent serum (14 days[a])	0, 0, 0	0, 0, 0	0, 0, 0	3, 2, 0	1, 3, 3	15, 16, 13	14, 16, 17
With acute serum (1 day)	14, 16, 17	16, 17, 19	18, 17, 20				
With Eagle's medium	17, 20, 19						

[a] Duration of rash when serum was taken.

RESULTS

1. *Serial cultivation of varicella virus (Oka strain) in GPEC*

After the 11th passage of Oka strain virus in HEL cells, infected cells were trypsinized and inoculated onto GPEC. Characteristic CPE appeared in a few days, and transfer of infected cell was repeated. Cell-free virus of 1,000 to 2,000 PFU/ml could be obtained from infected cells by sonication, but the viral yield varied depending upon the condition of cells, havesting time etc. Passaged virus was identified as varicella virus by hematoxylin-eosin staining (Fig. 3), fluorescent antibody staining (Fig. 4) and the neutralization test using HEL cells (Table 2).

TABLE 3. *Antibody response of children inoculated with various dose of varicella virus (Oka strain, HEL 11th passage, GPEC 6th passage)*

Case No.	Age (years)	Sex	Complement fixing antibody titer		
			pre	post	
	Virus dose		1,000 PFU		
1	4	M	<4	16	
2	8	M	<4	16	
3	2	M	<4	32	
4	3	F	<4	4	
5	4	F	<4	32	Mean $2^{4.0}$
6	10	F	<4	8	
7	5	M	<4	16	
8	4	F	<4	16	
9	5	M	<4	32	
10	5	F	<4	16	
	Virus dose		500 PFU		
1	4	M	<4	64	
2	6	F	<4	64	
3	3	M	<4	16	
4	2	M	<4	<4	
5	3	M	<4	8	
6	2	M	4	16	
7	1	F	<4	16	
8	1	F	<4	16	
9	6	F	<4	8	
10	8	F	<4	4	Mean $2^{3.8}$
11	3	M	<4	4	
12	1	M	<4	4	
13	1	M	<4	8	
14	7	M	<4	16	
15	5	M	<4	16	
16	5	M	<4	4	
17	4	F	<4	8	
18	3	M	<4	64	
19	2	F	<4	32	
20	2	F	<4	32	

2. *Vaccination of various doses of GPEC passaged virus*

After the 6th passage in GPEC, experimental vaccine was prepared and various doses of virus (1,000, 500, 200, 100 PFU in 0.5 ml per person) was given to groups of home-dwelling children with no history of varicella. With a dose of 1,000 PFU, seroconversion was positive in all 10 children tested. With 500 PFU, an antibody response was observed in

TABLE 3. *Continued*

Case No.	Age (years)	Sex	Complement fixing antibody titer		
			pre	post	
	Virus dose		200 PFU		
1	5	M	<4	4	
2	2	F	<4	64	
3	2	F	<4	4	
4	3	M	<4	8	
5	4	M	<4	64	Mean $2^{3.6}$
6	3	F	<4	8	
7	3	M	<4	16	
8	4	M	<4	8	
9	1	F	<4	4	
10	5	F	<4	16	
11	2	F	<4	16	
12	5	M	<4	<4	
	Virus dose		100 PFU		
1	1	M	<4	4	
2	3	F	<4	<4	
3	3	M	<4	4	
4	7	F	<4	16	
5	5	M	<4	32	Mean $2^{3.6}$
6	7	M	<4	4	
7	1	M	<4	<4	
8	3	F	<4	32	
9	2	F	<4	32	

19 of 20 children. Even with 200 PFU, an antibody response was detected in 11 of 12 children. When the viral dose was further reduced to 100 PFU, seroconversion was positive in 7 of 9 children (Table 3). The mean CF antibody titers of seropositive children in these groups were $2^{4.0}$, $2^{3.8}$, $2^{3.8}$ and $2^{3.6}$ respectively. No clinical symptoms due to vaccination were detected in the vaccinated children. These results showed that the Oka strain virus passaged 6 times in GPEC was well attenuated, and the minimum effective dose was about 200 PFU.

3. *Vaccination of various of virus propagated in WI-38 cells after passage in GPEC*

After the 12th passage in GPEC, the Oka strain of varicella virus was serially propagated in WI-38 cells, and was used for vaccination after the 2nd and 6th passages. With 200, 1,000, 2,000 PFU of virus after the 2nd passage, all vacciness in each group (9, 11 and 10 respectively) showed antibody responses and the mean CF antibody titers were $2^{4.0}$, $2^{4.4}$, and $2^{4.7}$, respectively (Table 4). No clinical reactions due to vaccination were

TABLE 4. *Antibody response of children inoculated with various doses of varicella virus (Oka strain, HEL 11th passage, GPEC 12th passage, WI-38 2nd passage)*

Case No.	Age (years)	Sex	Complement fixing antibody titer		
			pre	post	
	Virus dose		200 PFU		
1	5	F	<4	4	
2	1	F	<4	32	
3	5	M	<4	16	
4	3	F	<4	8	
5	4	M	<4	64	Mean $2^{4.0}$
6	3	F	<4	8	
7	2	F	<4	64	
8	3	M	<4	16	
9	3	M	<4	8	
	Virus dose		1,000 PFU		
1	2	M	<4	32	
2	4	M	<4	32	
3	1	F	<4	32	
4	7	M	<4	8	
5	4	F	<4	16	
6	4	M	<4	16	Mean $2^{4.4}$
7	1	M	<4	64	
8	5	M	<4	32	
9	5	M	<4	32	
10	1	M	<4	16	
11	5	F	<4	16	
	Virus dose		2,000 PFU		
1	4	M	<4	64	
2	6	F	<4	64	
3	3	M	<4	16	
4	3	M	<4	64	
5	2	F	<4	32	Mean $2^{4.7}$
6	2	F	<4	32	
7	4	M	<4	8	
8	1	F	<4	32	
9	2	M	<4	8	
10	1	F	<4	16	

observed. Ten children vaccinated with 500 PFU of virus after 6th passage also showed an antibody response without any clinical reactions (Table 5).

Thus it is evident that Oka strain of varicella virus propagated in WI-38 cells after passage in GPEC can also be used safely and effectively for vaccination of susceptible children.

TABLE 5. *Antibody response of children inoculated with varicella virus (Oka strain, HEL 11th passage, GPEC 12th passage, WI-38 6th passage)*

Case No.	Age (years)	Sex	Complement fixing antibody titer		
			pre	post	
	Virus dose		500 PFU		
1	2	F	<4	16	
2	5	M	<4	16	
3	2	F	<4	32	
4	3	M	<4	4	
5	2	M	<4	8	
6	4	F	<4	16	Mean $2^{3.9}$
7	3	F	<4	64	
8	4	M	<4	8	
9	2	M	<4	16	
10	5	M	<4	16	

DISCUSSION

Söltz-Szötz (1964, 1965) described the susceptibility of guinea-pig embryo cells to varicell virus. This was confirmed by Šefčovičová (1970), but cell free virus was not obtained. Fioretti et al. (1973) investigated the propagation of varicella virus in guinea-pig embryo cells in detail, and detected a small amount of cell-free virus. We cultivated the Oka strain of varicella virus serially in guinea-pig embryo cells and obtained a considerable amount of cell-free virus. After serial passage in GPEC, the virus was further propagated in human diploit cells (WI-38). Cell-free virus obtained either from infected GPEC or WI-38 cells was found to induce a satisfactory antibody response in susceptible children without causing detectable clinical reactions. Vaccine may be prepared with either GPEC or WI-38 cells. WI-38 cells are better because the virus yield is usually higher and no foreign cell proteins are present in the vaccine derived from these cells.

The antibody titers induced by the vaccine (mean CF antibody titer, 2^4) were generally lower than those induced by natural infection (usually 2^{7-9} CF antibody titer). However, the immunity due to vaccination should persist, and be boosted by inapparent natural infection if the antibody titer decreases. This has already been shown after measles vaccination (Ueda et al., 1970; Sangkawibha et al., 1971). Moreover, cellular immunity which may be afforded by vaccination should be significant in causing protection from infection (Russel et al. 1972). Follow-up studies are now in progress on the durability of immunuty induced by vaccination.

The causal relationship between varicella and herpes-zoster should be considered as there is a theory that herpes-zoster is caused by persistent varicella virus (Taylor-Robinson and Caunt, 1972). We don't know whether the fate of injected vaccine virus is the same as that of wild virus. However, the vaccine virus is too attenuated to cause any clinical symptoms, so even if it persists in the body, it is unlikely that it is converted to virulent virus which induces the apparent clinical symptoms of herpes-zoster.

The possible oncogenicity of varicella virus should also be considered, as this virus belongs to the herpes virus group. At present, it is unknown whether varicella virus is oncogenic, and this must be studied extensively. How-

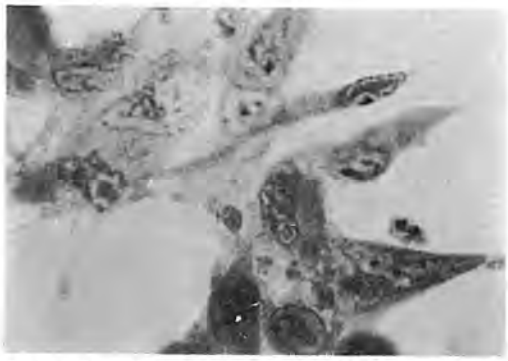

FIGURE 1. *Human embryo lung (HEL) cells infected with the Oka strain of varicella virus (at the 7th passage in HEL cells). 3 day after after infection. H-E staining.*

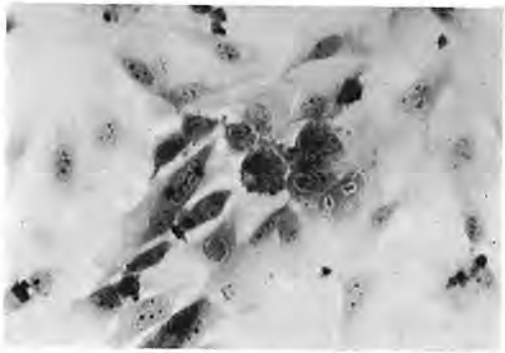

FIGURE 3. *Guinea-pig embryo (GPE) cells infected with the Oka strain of varicella virus (at the 6th passage in GPE cells after 11 passages in HEL cells). 3 days after infection. H-E staining.*

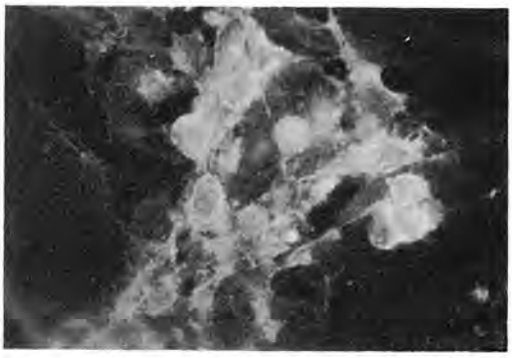

FIGURE 2. *HEL cells infected with the Oka strain of varicella virus (at the 7th passage in HEL cells). 3 days after infection. Fluorescent antibody staining (indirect method using varicella convalescent human serum and fluorescent antihuman rabbit serum).*

FIGURE 4. *GPE cells infected with the Oka strain of varicella virus (at the 6th passage in GPE cells after 11 passages in HEL cells). 3 days after infection. Fluorescent antibody staining.*

ever, wild varicella virus is usually present in nature, and most children are inevitably infected with it.

The clinical symptoms of varicella are usually mild in normal children, but in some cases, particularly in children with chronic diseases, varicella may be severe or even fatal. Moreover, recently, maternal varicella infection as a cause of fetal malformation and congenital varicella were reported and a safe and effective varicella vaccine is considered the most appropriate method to prevent this (Savage et al., 1974; Meyers, 1974). These considerations should be taken into account in evalution of the live varicella vaccine.

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FIGURE 5. *Negatively-stained electron micrograph of varicella virus particles of the Oka strain (prepared from the 5th passage in WI-38 cells after 12 passages in GPE cells).* ×*172,800.*

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