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Clinical and Laboratory Studies of KMcC Strain Live Attenuated Varicella Virus (41071)

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Abstract. Small-scale studies were carried out to test the KMcC strain varicella virus at various passage levels for clinical and serologic responses in order to find an optimal level of attenuation for vaccine purpose. Virus at passage 50 induced antibody responses, caused only minimal clinical reactions, and will be studied further. The clinical reactions to vaccine virus were very mild at all virus passage levels and were limited to mild transient fever and papular rash that became vesicular in a small proportion of individuals. The immune response to vaccination was accompanied by protection against natural challenge and antibody induced by vaccine virus persisted for at least 3.5 years, the longest period observed. Attenuation of virus appeared to be accompanied by alteration in the proportion of incomplete virus particles formed and by the degree of spontaneous release of virus from infected cells.

Herpesvirus varicellae or varicella-zoster virus is the cause of varicella (chickenpox) and zoster (shingles) in man (1). Varicella is one of the last remaining epidemic diseases of childhood. Though usually mild and benign, it can be severe or even fatal, especially in persons who are immunocompromised (2). Zoster occurs most frequently in elderly persons in whom the disease can be serious and life threatening. Zoster usually results from activation of latent varicella-zoster virus (recrudescence) during a period of waning immunity as in advanced cancer, or through use of immunosuppressive drugs (1–3).

The fact that immunity to chickenpox is long lasting and that the disease can be severe and fatal has stimulated interest in affording protection against the disease by vaccination. First attempts to vaccinate were carried out using vesicular fluid from human cases of the disease (4, 5). More recently, virus grown in cell cultures and released from cells by sonication (6, 7) has been employed in experimental vaccines (8–12).

Development of live virus vaccines consists of a series of small stepwise trials to determine the optimal balance between clinical reaction and immune response. The present report summarizes the findings to date in studies of reactogenicity and antibody responses to the KMcC strain of varicella virus attenuated by passage in WI-38 strain human diploid lung fibroblast cells in culture.

Materials and Methods. *Virus strain.* The KMcC strain of varicella virus was isolated by us in 1968, in WI-38 cell cultures, from frozen vesicle fluid of a male child who experienced varicella in 1966. Virus passage was carried out in WI-38 cell cultures. The first six virus passages were made in cultures incubated at 32°. Thereafter, passages of virus were made at 32 or 36° using infected cells to transfer the virus. The strain was identified as varicella by serum neutralization tests employing acute and convalescent sera from cases of clinical chickenpox in man and also using hyperimmune antiserum prepared in horses. Seven vaccines were prepared using virus passed from 10 to 60 times at 32 or 36° incubation temperature. A cloned line of varicella virus was developed by passage of progeny from a single plaque of virus (limit dilution) at passages 51, 52, and 53.

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This was passed seven more times to produce vaccine at passage level 60.

Cell cultures. The human diploid embryonic lung fibroblast (WI-38 strain) cell cultures that were used to pass the virus and to prepare vaccine were monitored according to recommended standards (13). The cell cultures were grown in standard cell culture medium to which irradiated fetal calf serum was added in 10% amount. During viral growth, the calf serum concentration was reduced to 2%.

Virus vaccines. WI-38 cell cultures in roller bottles were infected, 48–72 hr after planting, with varicella seed stock and incubated at 32 or 36°. Harvest was performed when 50% or more of the cells showed cytopathology. The overlying fluid medium was discarded and the cell sheets were rinsed four times with 100 ml of phosphate-buffered saline solution to reduce the amount of residual calf serum to less than one part per million. Sucrose–phosphate–glutamate–albumin stabilizer was added and the cell sheet was released by freezing and thawing. The cell suspension was treated by sonication to release the virus followed by filtration through a medium porosity (15 μm) sintered glass filter to remove residual cells. No intact cells were found on microscopic examination of a 50-fold concentration pellet obtained by centrifuging the vaccine filtrate. The vaccine filtrate was tested for safety, sterility, and freedom from adventitious agents according to the general standards for virus vaccines prescribed by the Bureau of Biologics, U.S. Food and Drug Administration (14). The tests included assays in monkeys inoculated intrathalamically, intraspinally, intracisternally, and intramuscularly, and examined histologically for lesions in the brain and spinal cord. Electron microscopy revealed the presence of both enveloped and nonenveloped (naked) herpesvirus particles in each preparation. Appropriate serum neutralization tests using paired human sera from cases of clinical chickenpox and animal hyperimmune sera were used to identify the virus in each vaccine lot.

Infectivity titrations. Viral infectivity titers were determined by plaque assay (15) on WI-38 cell cultures. The plaques were observed microscopically 7–8 days after inoculation and the counts were expressed as plaques per milliliter.

Antibody measurement. Varicella antibody in human sera was measured by the immune adherence hemagglutination assay (IAHA) (16–18) and by complement fixation (CF) (19) using commercially available antigens. Immunofluorescent antibody (FLAB) (20) and tube neutralization tests (21) in WI-38 cell cultures were also employed.

Electron microscopy. Virus particle counts were made using a thin sectioning technique described by Miller *et al.* (22). Complete (enveloped), incomplete (nonenveloped), and defective (enveloped virus without a core) particles were observed.

Clinical studies. The clinical trials of the vaccines were carried out from 1974 to 1979 in the Havertown area of suburban Philadelphia or in the environs of San Jose, Costa Rica. All vaccines were given with informed written consent and the studies were conducted in compliance with the Investigative New Drug regulations of the U.S. Food and Drug Administration. Children who were in overt good health, in the range of 1 to 8 years of age and having no prior clinical history of varicella were given 0.5 ml of the vaccine subcutaneously. Samples of blood for serologic determinations were taken prior to and 42 to 56 days after the vaccine was given. Temperatures were recorded daily by the parents or guardians during the 42- or 56-day period of observation. *In Costa Rica,* each child was seen on a daily basis in day-care or day-school centers by medical personnel. In the *Havertown studies,* each family was contacted by telephone on or about 3, 7, 16, 19, and 24 days after vaccination and home visits were made on or about Days 10, 12, 14, 21, and 28. In some of the Havertown studies, an additional child with a negative history of varicella was bled and retained for control purposes to permit measurement of protective efficacy of the vaccine. Surveillance for the occurrence of natural varicella in the families was made by par-

ent's and physician's reports. Suspect varicella rashes among the vaccinees and siblings were observed by a physician who obtained convalescent blood samples to confirm the diagnosis. Late bleedings were taken from a number of the vaccinees in the Havertown group to permit follow-up of the persistence of antibody. Clinical study P3 differed from the above in that it was carried out by Drs. Allan Arbeter and Stanley Plotkin among normal healthy children in the Philadelphia area.

Results. *Antibody responses in children.* Table I shows the antibody responses in children given vaccine of one of six different passage levels. The amount of virus ranged from 190 to 9500 PFU/0.5 ml dose. The sera from the vaccinated children were tested by the IAHA, FLAB, CF, and neutralization techniques. There was good agreement in the test results. The IAHA test proved to be the most sensitive and hence, was used routinely. All children who received virus in passage 10 through 40 developed antibody. The rates for seroconversion were less when virus at passage 45 and 50 was used. It was of importance, however, that all the children in study P3 developed antibody when measured in a highly sensitive fluorescent membrane assay (FAMA) performed by Dr. Arbeter at Children's Hospital of Philadelphia. None of the children who received the cloned virus at passage 60 developed antibody. The influence of virus dose on the antibody response was measured in tests summarized in Table II. It is seen that children given 60 or more PFU of virus at passage levels 10, 30, or 40 all seroconverted. Seven or 12 PFU were insufficient to immunize all the children.

Clinical findings. See Table III. Clinical illness resembling mild varicella developed in some of the children who were given the vaccine. The illnesses were self-limited and of short duration. Varicelliform rash was the most commonly present sign and this was usually macular or papular although small vesicles up to 5 mm in diameter were found present in some of the children. The numbers of lesions ranged from a single papule or vesicle at the injection site to 65 lesions found distributed on the head, neck, trunk, thighs, and arms. The frequency of rash decreased with increased passage of virus, when undiluted vaccine was given, indicating attenuation on passage. Lesion formation was increased by decreasing the virus dose, within the range of virus dosages capable of eliciting an antibody response. There was an increase both in the percentages of persons who developed generalized lesions and in persons who developed vesicles. No lesions were found in

TABLE I. ANTIBODY RESPONSES TO LIVE VARICELLA VIRUS VACCINES IN INITIALLY SERONEGATIVE CHILDREN

Vaccine virus		Clinical study		Serologic responses				
Passage level (36°)	Dose (PFU/0.5 ml)	No.	No. Vacc.	Seroconversion (IAHA) (%)	Geometric mean titer by assay			
					IAHA	FLAB	CF	Neut.
10	9500	385[a]	11	100	23	ND[b]	5	4
30	6000	427[a]	7	100	58	20	7	3
40	1150	548 and 556	38	100	41	18	8	4
45	900	575	10	90	23	ND	ND	ND
50	6500	547	6	83	11	16	<4	<2
		P3	17	94[c]	50	ND	ND	ND
60[d]	190	520	19	0	<2	<2	<4	<2

[a] Tests carried out in Costa Rica; all others in suburban Philadelphia or Havertown, Pa.
[b] Tests not done.
[c] All the children responded by the fluorescent membrane assay.
[d] Three passages, limit dilution (cloned).

TABLE II. INFLUENCE OF VIRUS DOSE ON ANTIBODY RESPONSES TO LIVE VARICELLA VIRUS VACCINE IN INITIALLY SERONEGATIVE CHILDREN

Vaccine virus		Clinical study		Serologic responses				
					Geometric mean titer by assay			
Passage (level)	Dose (PFU/0.5 ml)	No.	No. Vacc.	Seroconversion (IAHA) (%)	IAHA	FLAB	CF	Neut.
10[a]	7000	390 and 353	21	100	58	47	15	5
	700	390	11	100	155	100	34	10
	70	390	11	100	128	56	44	15
	7	390	10	70	39	32	23	9
30	6000	427[b]	7	100	58	20	7	3
	600	549	8	100	32	42	17	4
	60	427[b]	18	100	91	32	15	10
40	1150	556 and 548	38	100	41	18	8	4
	115	556	13	100	84	ND[c]	ND	ND
	12	556	13	69	23	ND	ND	ND
50	6500	547	6	83	11	16	<4	<2
	650	529	14	50	11	4	<4	<2

[a] Ten passages at 32°.
[b] Tests carried out in Costa Rica; all others in suburban Philadelphia.
[c] Tests not done.

TABLE III. CLINICAL FINDINGS IN INITIALLY SERONEGATIVE CHILDREN GIVEN LIVE VARICELLA VIRUS VACCINE

Vaccine virus		Clinical study		Findings					
				Rash (%)[a]				Onset rash (days)	
Passage level	Dose (PFU/0.5 ml)	No.	No. Vacc.	Total Group	Local	Generalized	Vesicular	Range	Average
10	9500	385	11	90	90	0	70	7–12	9
30	6000	427	7	71	71	0	14	8–12	9
40	1150	548 and 556	38	24	0	24	0	12–19	15
45	900	575	10	40	0	40	10	15–19	16
50	6500	547	6	0	0	0	0	—	—
		P3	17	12	0	12	0	14–18	16
60	190	520	19	0	0	0	0	—	—
10[b]	7000	390 and 353	21	52	29	24	0	7–14	10
	700	390	11	91	0	91	37	9–15	13
	70	390	11	82	0	82	46	12–20	15
	7	390	10	60	0	60	20	12–22	16
30	6000	427	7	71	71	0	14	8–12	9
	600	549	8	62	0	62	38	11–20	14
	60	427	18	6	6	0	6	7	7
40	1150	556 and 548	38	24	0	24	0	12–19	15
	115	556	13	62	0	62	8	11–22	15
	12	556	13	53	0	53	0	11–14	13
50	6500	547	6	0	0	0	0	—	—
	650	529	14	0	0	0	0	—	—

[a] Local = injection site only; generalized = other or additional area of skin.
[b] 32° passage. All others were 36°.

children who had received cloned passage 60 virus that was shown not to induce antibody.

Temperature was followed in all vaccinated children and fever, when present, was well tolerated. Sibling controls were observed in some of the studies and the findings in those investigations in which vaccinees and controls were included are shown in Table IV. Fever never exceeded 103.4°F and there was no real difference between vaccinees and controls when 40th or higher passage virus was given. There was more fever in vaccinees who received 10th or 30th passage virus than in controls, but the difference was small and fever did not exceed 102.4°F in these persons. Fever, when present, corresponded with the time that rash was present between Days 7 and 21 following vaccination.

Contagious transmission. Possible contagious transmission of varicella vaccine virus from vaccinated persons to susceptible sibling contacts was examined in two ways: development of varicelliform rash and serologic response. Blood samples were taken from the vaccinees and the sibling contacts 42 to 56 days after the vaccine was given. This should have allowed ample time for infection and antibody development in the contacts. Table V shows the findings in tests for development of varicella antibody among 84 seronegative children who resided in the same household in which at least one sibling who had been vaccinated showed evidence of infection based on development of antibody. No clinical varicella and no development of antibody occurred in any sibling contact except for two siblings in study 556. In the study, the vaccinated child developed mild varicella with papules and vesicles on Day 22 after vaccination. The two siblings contacts also developed mild varicella 36 days after the vaccine was given. All children showed very high IAHA antibody titers (vaccinee 128, sibling contacts 256 and 512)

TABLE V. TESTS FOR CONTAGIOUS TRANSMISSION OF KMcC VARICELLA VIRUS TO SUSCEPTIBLE CONTACTS

Vaccine passage level	Clinical study No.	No. seroconverted/total no.	
		Vaccinated	Seronegative contacts
36°			
10	385	11/11	0/6
30	427	12/12	0/13
30	549	8/8	0/5
40	548	23/23	0/22
40	556	18/18	1/18[a]
50	529, 547	11/11	0/12
32°			
10	353	9/9	0/8
Total		92/92	1/84

[a] One additional sibling, not initially enrolled in study 556, also developed varicella.

TABLE IV. TEMPERATURE ELEVATIONS (≥100° F, ORAL) AMONG INITIALLY SERONEGATIVE CHILDREN WHO WERE GIVEN VARICELLA VACCINE OR WHO WERE SIBLING CONTROLS

Vaccine virus		Clinical study			Number with fever, Days				Maximum fever Days 7–21
Passage level	Dose (PFU/0.5 ml)	No.	No. Vacc.	No. Control	0–6	7–13	14–21	22–28	
10[a]	7000	353	10		1	3	2	0	102.4
				10	1	1	0	0	100.2
30	650	549	8		2	4	3	0	101.0
				5	0	1	0	0	100.0
40	1150	548	25		4	4	6	5	103.0
				24	4	4	3	4	103.0
50	6500	547	6		0	2	0	0	103.0
				6	0	1	1	1	100.2
50	650	529	14		1	0	1	1	102.2
				13	0	1	1	0	102.4
60	190	520	19		0	0	0	0	103.4
				17	1	3	1	3	103.4

[a] 32° passage virus. All other vaccine viruses were passed at 36°.

in their second serum samples. Varicella virus was recovered from vesicle fluid of all three children. Whether contagious transmission had occurred to the two siblings cannot be stated since natural varicella was occurring in the community at the time.

Protective efficacy of varicella vaccine. Clinical varicella occurred in 11 of the families in which there was a vaccinated child who responded serologically to vaccination and in which one or more siblings developed natural varicella 6 to 143 weeks after the time the vaccine was given. All cases were proved serologically. The findings are shown in Table VI. Thirteen initially seronegative children, all of whom had developed varicella antibody after vaccination, were put at risk to 17 cases of natural varicella that occurred in their families. All the vaccinated children were protected except for one child who had received vaccine virus at 50th passage. The virus had been diluted 1:10 and 650 PFU of virus had been given. The IAHA antibody titer in this child following vaccination was 1:8 and the case was proved serologically. The illness was afebrile, extremely mild, and consisted of a generalized varicelliform rash consisting of 26 lesions with vesicles and crusts.

Persistence of antibody following vaccination. Seven children who responded serologically 2 months following passage 10 (some 32 and some 36°) vaccine were bled again 2.5 to 3.5 years later. Most of these seven children were exposed to natural varicella in their neighborhood or schools but none developed the disease. Twelve children who responded serologically 2 months following passage 40 vaccine were bled again 6 months to 1 year later. Only two of these children were exposed to natural varicella in their schools. The 2-month and later bleedings were tested at the same time for varicella antibody by the IAHA test and the findings are shown in Fig. 1. All children in both study groups retained varicella antibody but there was an up to 32-fold decline in the titer of antibody in the late sample. The mean antibody titer declined 3.6-fold for passage 10 vaccine during the following 2.5–3.5 years and the mean titer declined 5.4-fold for passage 40 vaccine during 6 months to 1 year after the vaccine was given. These declines in height of antibody were substantial in some of the children and it is not possible at this time to predict how long immunity may persist.

Electron microscopic examination of the virus. Morphologic and biologic attributes of the KMcC virus at three passage levels were determined and comparisons were made with those of virus in vesicle fluid from natural cases of varicella. As shown in Table VII, cell-free KMcC virus showed progressive increase in incomplete and defective forms from 56% at passage 10 to 71% at passage 60. Natural varicella virus from vesicle fluid had only 42% of incomplete or defective particles. Most of the virus seen in cut sections of the infected cells were complete, containing a core, but the percentage of complete virus particles was less for the cloned 60th passage virus. The yields for infectious virus from soni-

TABLE VI. PROTECTION AGAINST VARICELLA IN NATURE AMONG VACCINEES WHO HAD DEVELOPED ANTIBODY FOLLOWING VACCINATION

Vaccine passage level	Clinical study No.	Vaccinated (cases/total)	Natural varicella (No. of cases)[a]
36°			
40	548	0/2	3
40	556	0/1	1
50	529	1/2	5
32°			
10	353	0/3	3
10	390	0/5	5

[a] No. of cases of varicella in nonvaccinated children in same families as the vaccinees.

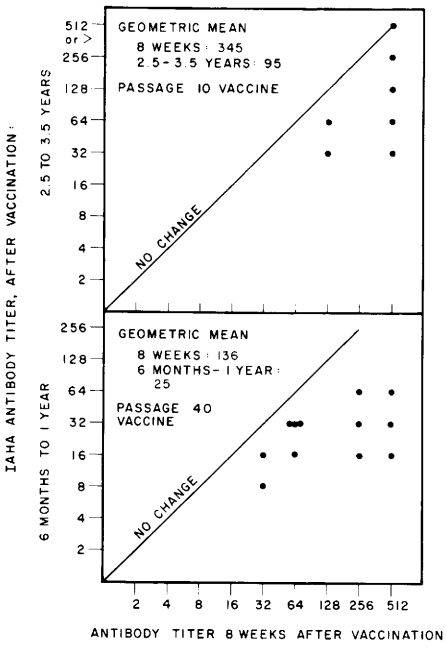

FIG. 1. Persistence of varicella antibody (IAHA) following initial 8-week response to vaccine at passage levels 10 and 40.

cated cells were not markedly different for the three passage levels (2.3 to 14 × 10^3) but the amount of virus released spontaneously from the infected cells was strikingly greater for the cloned passage 60 virus compared with the 10th or 40th passage virus.

Discussion. An ideal attenuated varicella virus vaccine should: (a) cause little or no clinical reaction, (b) lack the capability for contagious spread, (c) induce antibody that is persistent, and (d) protect against the natural disease. The present findings show that there is attenuation for varicella virus on serial passage in cell culture with decrease in clinical reactions and decrease in antibody response. Reduced amount of virus, within the effective dose range, often gave increased clinical reactogenicity and increased amount of antibody. There is no definitive explanation for this but it is possible that the larger virus dose might have contained sufficient viral substance to induce an early local and cell-mediated immunity that might have aborted or at least

TABLE VII. MORPHOLOGY AND INFECTIVITY OF CELL CULTURE PROPAGATED KMcC VIRUS WITH VIRUS FROM VESICULAR FLUID OF A CASE OF VARICELLA

Measurement	KMcC virus at passage			Varicella virus in nature
	10	40	60	
Electron microscopy				
Cell-free virus (particles/ml)				
Total virus[a]	70 × 10^8	34 × 10^8	14 × 10^8	5.9 × 10^8
Complete virus	31 × 10^8	13 × 10^8	3.4 × 10^8	3.4 × 10^8
Percentage incomplete or defective	56%	63%	71%	42%
Infected cells				
Cut section (particles/field)				
Total virus[a]	33	22.4	11.9	—
Complete virus	9	2.4	6.2	—
Percentage incomplete or defective	73%	89%	48%	—
Infectivity (PFU/ml)				
Sonicate[b]	14 × 10^3	2.3 × 10^3	3.8 × 10^3	—
Spontaneously released[c]	$<10^1$	$\sim 10^1$	10^2–10^3	$\sim 10^2$

[a] Complete virus plus incomplete and defective virus.
[b] Cell-free virus.
[c] Filtered infected culture fluid.

decreased the extent of viral proliferation. Whatever the degree of clinical reaction, it was mild, self-contained, and inconsequential to the recipients. Fever, when present, was tolerated well by the children and was accepted by the parents. Rash, when present, was usually papular but progressed to vesicle formation in some children and this was most frequent with lower passage virus. The vaccine has not been tested to date in immunocompromised persons. Contagious transmission of vaccine virus from vaccinated to susceptible persons appears to occur rarely, if at all. The two cases that were observed in contacts in the present studies might have resulted from intercurrent infection, but this remains to be proved.

The vaccine at the serial passage levels tested clearly induced antibody and afforded protection against the disease on natural challenge. The single case of varicella that occurred in a vaccinee was in a child who received 650 PFU of virus at 50th passage and his antibody response was only to 1:8 titer. Antibody persisted in all the subjects tested up to 3.5 years. The pattern for decline of antibody is not unlike that for other virus infections such as measles, mumps, and rubella (23) that show an early substantial drop in titer with persistence at the same level thereafter. The length of persistence of antibody following varicella vaccine will await a longer period of observation.

Attenuation of virulence of varicella virus for man was accompanied in the present studies by morphologic alteration of the virus. With increased passage, the more attenuated cell-free virus, especially the cloned passage 60 virus, appeared to have a greater proportion of incomplete virus particles, and greater spontaneous release of virus from the infected cells. These findings, if confirmed, may be of value in guiding vaccine virus attenuation and in providing markers for attenuation of vaccine virus strains.

Vaccine prepared from cloned virus at passage 60 is clearly overattenuated as judged by absence of antibody response. The 50th passage virus may be sufficiently infectious to provide protection while causing only minimal reactivity and no apparent problem of contagious transmission. Expanded clinical trials and longer periods of observation of the children receiving this vaccine are clearly justified to determine its potential as a candidate vaccine strain.

It is possible that attenuated vaccine virus will establish persistent infections and that recrudescence of vaccine virus will also occur. It might be anticipated that the lesions resulting from the attenuated virus will be less than those from virulent virus and it may be speculated that zoster-like illness, if any, will be substantially less than in persons who are infected with the virus in nature.

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