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Candidate Cytomegalovirus Strain for Human Vaccination

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A strain of human cytomegalovirus called Towne was isolated in WI-38 human fibroblast cell cultures from the urine of an infected infant. It was then passaged 125 times in WI-38, including three clonings, and a pool was prepared in the same cell substrate for use as a potential live attenuated vaccine. The Towne virus has a broad antigenicity and cross-reacts with the AD-169 strain. Several markers of the Towne virus were found which differentiated it from fresh isolates. One of these was resistance of the former to trypsin. The Towne virus was tested for freedom from oncogenicity or other harmful effects in preparation for tests in humans.

The incidence of intrauterine cytomegalovirus (CMV) infection in developed countries is about 1% (27). Of the infected neonates, at least 10% suffer some brain damage (15) and a greater proportion sustain some degree of hearing loss (24). In older populations, the syndrome of CMV infection after large-volume blood transfusion is a frequent and troublesome complication of cardiac surgery and exchange transfusion (3, 8, 11, 20, 21, 23). Although the immunology of CMV infection is incompletely understood, and it is uncertain whether or not fetal infection is always the result of primary infection, it seemed prudent to begin efforts to develop a prophylactic that could be administered to prepubertal girls or to women prior to pregnancy, or to recipients of large-volume blood transfusions.

The possible objections to this decision to go ahead with research on human vaccination against CMV are presented in the Discussion.

Elek and Stern (9) have already reported the immunization of adult volunteers with a tissue culture passage of CMV strain AD-169, originally recovered from adenoidal tissue. They used a variety of human cell strains in the course of passaging their virus. We have also been pursuing the development of an attenuated CMV strain, but have taken a somewhat different approach from that of Elek and Stern. We started with a fresh isolate and passaged it solely in WI-38 human diploid fibroblasts, a cell substrate now used for the production of a variety of vaccines. An extended series (over 125) of passages was made during which the virus was cloned three times by end point dilution passage before experimental administration to humans.

MATERIALS AND METHODS

CF antigen and precipitin antigen. Infected WI-38 monolayers were suspended in 0.25% trypsin, and the resulting cell suspensions were washed twice in phosphate-buffered saline to make 5% cell suspensions. These cell suspensions were disrupted by sonication for 2 min in a Raytheon sonic oscillator at 1 A and centrifuged at 2,000 rpm for 10 min. The resulting supernatant fluid was used as antigen in complement fixation (CF) or immunoprecipitin tests. The titer of the CF antigen was determined by checkerboard titration against a known positive serum. Control antigen from uninfected WI-38 cells was similarly prepared.

CF test. The test was carried out using the microtiter technique (25). Two units of complement, 2 U of hemolysin, and a 2% suspension of sheep erythrocytes were used in the test.

Gel-precipitin test. Molten agarose (0.4%) dissolved in Ca- and Mg-free phosphate-buffered saline, pH 7.2, containng 0.01% merthiolate was added in 25-ml amounts to 9-cm glass petri dishes. Wells (8 mm in diameter) were made with centers 12 mm apart. The center well was filled with antiserum, and the adjacent wells were filled with antigen. The dishes were incubated in moist boxes at 4 C for 1 week. Precipitin lines were observed under illumination.

Infectivity assay. Viral infectivity titers were determined in cultures of WI-38 cells by means of cytopathic effect (CPE) or plaque assay. The 50% end points for CPE were calculated by the method of Reed and Muench (22). The plaque assay method was that of Wentworth and French involving WI-38 cells plaqued under agarose (31).

Infectious center assay. Monolayers were suspended by trypsin, and the cells were dispersed by pipetting. An aliquot was removed for a hemocytometer cell count, and the remainder was diluted in serial 10-fold dilutions. Each dilution (0.2 ml) was inoculated onto three WI-38 monolayer cultures in petri dishes. After 7 days foci were counted by examination of stained dishes under the microscope.

Trypsin sensitivity test. Infected cultures were harvested when the CPE was 80%. Sonicated cells and the culture fluids were mixed and centrifuged at 6,000 rpm for 30 min. The supernatants were then sedimented at 22,000 rpm for 60 min. The pellets containing virus were resuspended in serum-free minimal essential medium. One and eight-tenths milliliter of virus in serum-free minimal essential medium was mixed with 0.2 ml of 2.5% trypsin (Difco) and incubated at room temperature or at 37 C for various lengths of time. To inactivate the trypsin, at the end of the prescribed intervals calf serum was added to give a final concentration of 10%. The suspension was then titrated by plaque assay.

Virus strains. The origin of the Towne strain is described under Results. The other CMV strains mentioned in this paper were isolated from the urine of congenitally infected infants. The isolates were identified as CMV by a topical CPE on WI-38 human fibroblasts and by CF reaction against standard sera. The isolates were serially propagated on WI-38 in our laboratory, starting when CPE involved over 50% of the cells.

RESULTS

Derivation of the Towne strain. The Towne strain of CMV was isolated in March 1970 from a 2-month-old infant with microcephaly and hepatosplenomegaly. The virus was isolated from the urine directly into a WI-38 cell culture. The child is now severely retarded but still alive. At the time of the initial diagnosis, he manifested an antibody response against AD-169 CF antigen. The original virus gave a typical CPE for CMV, and intranuclear inclusions were visualized on stained preparations. A CF antigen prepared from the virus gave appropriate reactions when tested against sera previously known to be positive or negative when tested against AD-169 antigen.

The first 10 passages of this virus were accomplished by suspension of infected cell monolayers in trypsin and the transfer of 25% of the infected cells to each of four new WI-38 cultures. The incubation temperature was 37 C. The next 28 passages were accomplished by the passage of 1 ml of supernatant tissue culture fluid per new monolayer WI-38 culture. With adaptation, 10^6 or more plaque-forming units (PFU) of virus were released into the tissue culture fluid when the cells were infected at a high multiplicity of infection (≥ 1) and when the fluid was harvested after 100% of the cells were visibly involved. The growth curve of this infection (Fig. 1) reveals a peak production of cell-associated virus at 4 days, but cell-free virus at 7 days.

In subsequent passages, except for the ones to be mentioned specifically, virus released from sonicated cells was added to the supernatant virus and the combined suspension was used as inoculum.

At passages 50, 60, and 70, the inoculum was diluted terminally, and the dilution containing one isolated focus of infection was used as the subsequent inoculum.

Preparation of virus pool. The Towne strain was sent to Recherches et Industries Therapeutiques laboratories at passage 117. Passage of cell-free virus was made there until passage 125, at which time the virus was frozen as seed virus. WI-38 cultures were inoculated with the seed virus, using maintenance medium without serum or antibiotics. Supernatant fluids harvested at 7 days were filtered through a 3-μm filter combined with a stabilizer and then lyophilized. Two batches of experimental vaccine were prepared at passage 128. They were freeze dried, and both were tested for safety and potency as described below.

The lyophilized virus was stable at 4 C or lower. After reconstitution the titer was $10^{3.0}$ PFU/ml for batch 1 and $10^{4.7}$ PFU/ml for batch 2.

Neutralization of virus. Cell-free Towne virus could be completely neutralized by various antisera, including: human CMV-convalescent serum sold by Flow laboratories (CF titer 1/16) and diluted 1/10; hyperimmune guinea pig serum against AD-169; and hyperimmune rabbit serum against Towne, prepared by inoculation of 1 ml subcutaneously (10^6 PFU) at 1-week intervals for five inoculations.

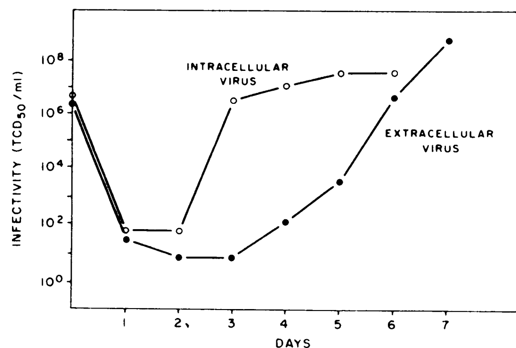

FIG. 1. *WI-38 confluent monolayers (T-25 Falcon flask containing approximately 10^6 cells) were infected with CMV Towne strain at a multiplicity of 10. At various intervals indicated in the figure the tissue culture medium was removed, and the cells were trypsinized and resuspended in 2 ml of media. The cell suspensions were then subjected to sonication for 2 min. After centrifugation for 10 min at 2,000 rpm, the supernatants were titrated for infectivity, as designated "cell-associated virus." The tissue culture fluids previously removed were also centrifuged and titrated to determine the "cell-free virus."*

Table 1 summarizes the serum neutralization titers alone and in the presence of complement.

Safety tests. Safety tests of a stock solution of the virus pool in animals are shown in Table 2. In addition to these tests in animals, 50 ml of undiluted virus pool was tested for adventitious agents in cell cultures from four sources: macaca monkey kidney, cercopithecus monkey kidney, primary rabbit kidney, and human kidney. Each of the cell cultures was observed for 14 days. At the end of the observation period at least one subculture of fluid was made in the same tissue culture system, and the subcultures were observed for 14 days. The tissue cultures failed to show evidence of the presence of simian viruses or any other viral agents, including hemadsorbing viruses.

TABLE 1. *Neutralization to Towne virus*

Serum sample	Neutralization by serum[a]	
	Alone	With C′
Rabbit		
no. 1	<8[b]	64
no. 2	<8	32
no. 3	<8	64
Guinea pig (U. Krech)	20–100	100
Human antiserum (Flow Laboratories)	40	40

[a] Serum neutralization was done in microplates (Linbro): 0.025 ml of suspension of 25 to 60 tissue culture infectious doses of Towne 125 plus 0.025 ml of diluted serum. After 1 h, 0.250 ml containing 10^4 WI-38 cells in Eagle growth medium with 10% unheated calf serum was added. Microplaques were read after 5 to 7 days of incubation at 37 C. Eight wells per dilution were used. The titers were generally higher in the presence of complement, but complement alone had no neutralizing effect.

[b] Reciprocal of titer.

The presence of mycoplasma in the bulk vaccine pool was tested in agar and semisolid medium. Each test also included at least two cultures of known strains, one of which was *Mycoplasma pneumoniae*. One-half of the culture tubes was incubated aerobically, and the other half anaerobically, for 14 days and observed for growth of mycoplasma. At days 3 and 14, two tubes in each instance were subinoculated and observed for another 14 days. The vaccine showed no evidence of mycoplasma.

Each bulk virus pool was tested for bacterial sterility in fluid thioglycolate for aerobic and anerobic bacteria, in Lowenstein-Jensen agar slants, and in modified Dubos broth (TB broth base [Difco], bovine serum dextrose, and glycerol) for *Mycobacterium tuberculosis*. There was no evidence of microbial contamination.

In addition, 20 cercopithecus monkeys were inoculated with each batch of the Towne 125 strain, 0.5 ml intraspinally, 1 ml intracerebrally, and 1 ml intramuscularly. Monkeys used for the first batch were all sero-negative when tested in a neutralization test using 100 mean tissue culture infectious doses of CMV (Towne strain) both in the absence and presence of 1 U of fresh guinea pig complement. These monkeys were tested again by the same method 3 weeks later, and no seroconversion was demonstrated. For a second batch, prepared from the same pool, another group of monolayers was inoculated. However, the seroneutralization test used to check the serological status of the second

TABLE 2. *Safety tests in animals for each of two batches of Towne virus*

Animal	No. (ind. wt)	Inoculum		Observation time (days)	Results
		ml	Route[a]		
Adult mouse	20	0.5 0.3	i.p. i.c.	21	No disease or pathology
Suckling mouse	20	0.1 0.01	i.p. i.c.	14[b]	No disease or pathology
Guinea pig	5 (350–450 g)	5.0 0.1	i.p. i.c.	42	No disease or pathology
Rabbits	10 (1,500–2,500 g)	1.0 9.0	i.d.[c] s.c.	30	No disease or pathology
Monkeys	20	1.0 0.5 1.0	i.c. i.s. i.m.	21	No disease or pathology

[a] i.p., Intraperitoneal; i.c., intracerebral; i.d., intradermal; s.c., subcutaneous; i.s., intraspinal; i.m., intramuscular.

[b] Surviving mice were pooled, emulsified without skin and viscera, and passed blindly in five mice i.c. and i.p.

[c] In multiple sites.

group of monkeys prior to inoculation and 17 to 19 days after inoculation was modified. Five units of fresh guinea pig complement was used, and the neutralization was carried out at 37 C instead of at room temperature. Under these conditions, four monkeys were completely seronegative before inoculation and 16 presented titers ranging from 2 to 4. A fourfold or greater increase in titer was observed in 18 monkeys among 20. There were no signs of neurological disease in any of these 40 monkeys after clinical and pathological examination.

Cross neutralization of human CMV by green monkey and baboon sera positive for simian CMV has been reported by Minamishima et al. (17). These cross-reactions were only observed in the presence of complement. The low amount of complement added to the serological test on monkeys inoculated with the first batch and the neutralization at room temperature might be responsible for the absence of detectable seroconversion, but it was not possible to repeat the study owing to insufficient serum.

Antigenic spectrum of Towne. The Towne strain appears to be related to AD-169 in the breadth of its reactivity. Two rabbit antisera to Towne each gave titers of 1/32 in a CF reaction against AD-169 or Towne. Both sera also produced precipitin lines against both antigens. These sera were produced by five weekly inoculations of partially purified virus (1 ml of a sucrose density gradient fraction containing 10^7 PFU plus 1 ml of complete Freund adjuvant). The sucrose gradient was prepared as described previously (10). When negative and positive human sera were tested against 2 U of antigens prepared from Towne or AD-169, the results were virtually identical (Table 3). Testing showed common precipitin and CF antigens between Towne and four other strains when tested against human antisera (Fig. 2). CF antigens prepared from one low-passage fresh isolate (5386), AD-169, and Towne, when tested against an antiserum raised in rabbits against Towne virus, showed cross-reactivity. Two units of each antigen gave a serum titer of 1/128.

TABLE 3. *Comparison of CF titers using AD-169 and Towne strain antigens*

Human sera	AD-169	Towne
S 1	8	16
S 2	128	128
S 3	32	64
S 4	<4	<4
S 5	64	64
S 6	<4	<4
S 7	32	64
S 8	4	8
S 9	<4	<4

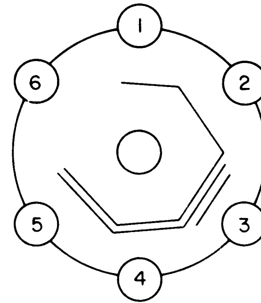

FIG. 2. *Diagram of precipitin lines. Human convalescent serum was in the center well; the peripheral wells contained Towne virus, four low-passage fresh isolates, or extract of normal WI-38 cells.*

Markers. With respect to markers of attenuation, it would be incorrect to say that there are any known "in vitro correlates" of "in vivo attenuation." However, there are several promising markers of identity. The clearest is the ratio of virus released into the supernatant to the number of infected cells. With the high-passage strain, an average of 0.1 to 1.0 infectious units per infected cell (as measured by infectious center assay) is easily achieved. Low-passage fresh isolates, in our experience, do not release an average of more than 0.001 infectious virus per infected cell.

A second marker is thermostability. Both Towne and AD-169 are stable for 24 h at 4 C, whereas C-87, Davis, and Kerr all have paradoxical instability at that temperature (29).

Third, Kim and Carp (14) have recently reported that CMV strains Towne, AD-169, Davis, Espaillat, and UW are all resistant to 0.25% trypsin. We have confirmed that observation with Towne and have gone on to test other low- and high-passage strains. Low-passage viruses were decidedly more sensitive to 30 min of incubation in 0.25% trypsin at room temperature (Table 4). The same phenomenon was observed at 37 C.

Test for oncogenicity. With respect to possible oncogenicity, four sorts of tests have been performed. The first was to inoculate live high-titer CMV into suckling mice treated with antilymphocyte serum. No tumors or mortality (except for deaths due to bacterial infection) were seen.

The second was to attempt to repeat Albrecht and Rapp's (1) work in hamster embryo cultures. High-titer live Towne virus (10^7 mean tissue cultures infectious doses/ml) and Towne virus inactivated with ultraviolet light for various periods of time up to 10 min were inoculated onto hamster embryo fibroblasts, which were

TABLE 4. *Trypsin (0.25%) sensitivity of various CMV strains at room temperature*

Name of strain	Passage level	Original titer (log 10)	Loss of titer (log 10)	
			30 min	60 min
High	9	5.00	−1.00	−2.00
High	20	6.00	−0.00	−0.00
5269	8	6.44	−0.97	−1.21
5386	4	5.32	−0.94	−1.02
5419	4	3.65	−2.00	−2.00
Towne	123	6.04	−0.08	−0.20
MA-420 (HSV)[a]		7.00	−3.5	−3.5

[a] HSV, Herpes simplex virus type 1.

then maintained for 3 months. The unirradiated virus caused a temporary CPE, but no foci of transformation occurred in those cultures or in those inoculated with ultraviolet-irradiated virus.

Third, using the technique of Darai and Munk (25), WI-38 was infected with Towne and maintained at 42 C for 1 or 2 weeks. Subsequent transfer of cultures to 37 C resulted in morphological changes associated with viral growth, but no transformants were rescued.

Fourth, hamsters inoculated at birth with $10^{6.5}$ mean tissue culture infectious doses of Towne 125 virus, either untreated or irradiated with ultraviolet light for 1, 3, 5, or 10 min, showed no tumors after 3 months of observation.

Fifth, 51 1-day-old hamsters were inoculated subcutaneously with material from batch 1; they have now been under observation for 12 to 13 months. No tumors have been observed in any of the animals. An identical number of hamsters were also inoculated by the same route with batch 2 of vaccine; they remain negative for tumors after 5 to 6 months.

DISCUSSION

The Towne strain of CMV at passage 128 was chosen for testing in humans because it seemed reasonable that high passage in tissue culture had attenuated its pathogenicity. Several markers differentiated the high-passage virus from wild strains, including greater release of extracellular virus (13) and trypsin resistance. In the absence of an experimental animal, only the development of modified CMV strains and actual testing in humans can determine whether attenuation has been achieved.

Objections to the development of attenuated vaccines to prevent fetal CMV infection fall under the headings of safety and efficacy. The questions of safety are three: (i) immediate reactions, (ii) possible oncogencity, and (iii) possible persistence of attenuated CMV in a latent stage. This paper cannot consider these issues in detail, but some comments are pertinent.

The Towne strain has been given intranasally and subcutaneously to adult volunteers. No infection resulted from intranasal administration to 18 volunteers. Subcutaneous inoculation of 10 volunteers produced antibody responses, but no central effects apart from local reaction and lymphycytosis in some subjects. General health remained good, and no virus excretion was detected (12a; S. A. Plotkin and J. D. Farquhar, unpublished data).

The main reasons for concern about the oncogenicity of CMV is the report of its transformation of hamster embryo cells by Albrecht and Rapp (1) and the apparent oncogenicity of some other herpes group viruses. The hamster cell transformation data are difficult to interpret in view of their having been acquired with ultraviolet-irradiated virus and in view of the transformation ability of other viruses which are apparently not oncogenic for humans (e.g., adenoviruses and simian virus 40). Although natural Marek's disease, Epstein-Barr virus, *Herpesvirus saimiri*, and herpes simplex type 2 are all possible or probable oncogenic viruses, it is reassuring to note that serial passage of Marek's disease virus resulted in a strain that was nononcogenic (4). Moreover, many live herpes group viruses indigenous to lower animals have been used extensively for veterinary vaccination, such as infectious bovine rhinotracheitis. The U.S. Department of Agriculture estimated that 72 million doses had been given in 1973 alone.

No evidence for oncogenicity of CMV in humans has been produced, although it is estimated that 6% of all units of transfused blood contain CMV (8, 11, 21). Lang et al. (16) have reported some growth in agarose of human cells infected with CMV, but were unable to obtain transformants. Their results therefore remain enigmatic.

Persistence and possible later reactivation of latent attenuated CMV cannot be excluded as a possibility at this time, although seroepidemiological studies demonstrate that natural CMV infection can be expected to occur in virtually everyone by the later years of life (7, 30). We hypothesize that an attenuated virus originally having restricted multiplication would be no more likely to reactivate than virus from a natural infection.

The questions relating to efficacy are three: (i) Does fetal infection result from primary exposure of the mother or reactivation of preceding infection? (ii) Will CMV strains cross-protect against each other? (iii) Will attenuated strains protect against disease caused by wild viruses?

The seroepidemiological studies mentioned above (7, 30) show that at least a third of young women have had no prior exposure to CMV. A prospective study (18) and a single case report (6) testify to the risk to the fetus of primary infection of the mother during pregnancy. On the other hand, three instances of repeated infections in subsequent pregnancies of seropositive mothers have been reported: nevertheless, it is encouraging to note that in contrast to the first infection, which was symptomatic, the second infected siblings remained asymptomatic (26).

Secondly, despite acknowledged strain differences, there is much evidence of cross-antigenicity (2, 12). Actual observations of natural or artificial challenge with heterologous strains will be necessary to settle the importance of strain differences.

With regard to the question on efficacy of attenuated vaccines, at this point only surmise by analogy is possible. Osborn and Walker (19) showed that a partially attenuated strain of mouse CMV protected inoculated mice against later challenge. We propose in future experiments to test the hypotheses that protection against fetal infection by CMV can be elicited by prior vaccination and that the cellular immunity induced by a living virus will be necessary to provide that protection.

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