

A Phase 1 Study of 4 Live, Recombinant Human Cytomegalovirus Towne/Toledo Chimera Vaccines in Cytomegalovirus–Seronegative Men

Stuart P. Adler,¹ Anne-Marie Manganello,¹ Ronzo Lee,² Michael A. McVoy,² Daniel E. Nixon,³ Stanley Plotkin,^{4,5} Edward Mocarski,⁶ Josephine H. Cox,⁷ Patricia E. Fast,⁷ Pavlo A. Nesterenko,⁸ Susan E. Murray,^{8,9} Ann B. Hill,⁸ and George Kemble¹⁰

¹CMV Research Foundation, ²Department of Pediatrics, and ³Department of Internal Medicine, Virginia Commonwealth University, Richmond; ⁴University of Pennsylvania, and ⁵Wistar Institute, Philadelphia, Pennsylvania; ⁶Department of Microbiology and Immunology, Emory Vaccine Center, Emory University, Atlanta, Georgia; ⁷International AIDS Vaccine Initiative, New York; ⁸Department of Molecular Microbiology and Immunology, Oregon Health and Science University, and ⁹Department of Biology, University of Portland, Oregon; and ¹⁰3-V Biosciences, Menlo Park, California

(See the editorial commentary by Demmler Harrison on pages 1297–9.)

Background. Human cytomegalovirus (HCMV) infection causes disease in newborns and transplant recipients. A HCMV vaccine (Towne) protects transplant recipients.

Methods. The genomes of Towne and the nonattenuated Toledo strain were recombined, yielding 4 Towne/Toledo chimera vaccines. Each of 36 HCMV-seronegative men received 1 subcutaneous dose of 10, 100, or 1000 plaque-forming units (PFU) in cohorts of 3. Safety and immunogenicity were evaluated over 12 weeks after immunization and for 52 weeks for those who seroconverted.

Results. There were no serious local or systemic reactions. No subject had HCMV in urine or saliva. For chimera 3, none of 9 subjects seroconverted. For chimera 1, 1 of 9 seroconverted (the seroconverter received 100 PFU). For chimera 2, 3 subjects seroconverted (1 received 100 PFU, and 2 received 1000 PFU). For chimera 4, 7 subjects seroconverted (1 received 10 PFU, 3 received 100 PFU, and 3 received 1000 PFU). All 11 seroconverters developed low but detectable levels of neutralizing activity. CD4⁺ T-cell responses were detectable in 1 subject (who received 100 PFU of chimera 4). Seven subjects receiving chimera 2 or 4 had detectable CD8⁺ T-cell responses to IE1; 3 responded to 1–2 additional antigens.

Conclusions. The Towne/Toledo chimera vaccine candidates were well tolerated and were not excreted. Additional human trials of chimeras 2 and 4 are appropriate.

Clinical Trials Registration. NCT01195571.

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A primary human cytomegalovirus (HCMV) infection during pregnancy causes congenital mental retardation and deafness in approximately 4000–9000 newborns annually in the United States [1]. For this reason and because of the cost associated with caring for those affected by HCMV, 17 years ago an Institute of Medicine report gave the highest priority to the development of an HCMV vaccine [2]. Despite this report and subsequent efforts by many, no HCMV vaccine is available, and none are in late-phase trials, although many candidates are being developed [3].

Work on an HCMV vaccine began nearly 40 years ago, with live attenuated HCMV vaccines [4, 5]. Of these, the Towne vaccine is the most comprehensively studied. Towne vaccine produces no local or systemic reactions and has an unflawed safety

record [6–11]. The Towne strain does not appear in the blood, urine, or saliva after vaccination, suggesting that the vaccine does not cause a systemic infection.

Seronegative renal transplant recipients vaccinated with the Towne strain were partially protected against HCMV disease but not against infection with wild-type virus [12–14]. A low dose of the Towne strain, unlike natural HCMV infection, did not protect immunocompetent seronegative women from wild-type infections [15]. Towne vaccine recipients were partially protected against HCMV disease and infection following experimental challenge with low-passage Toledo strain, but the level of the protection afforded by the vaccine was less than that of wild-type infection [16].

These observations suggest that Towne vaccine may not prevent HCMV infection. If true, the Towne strain may be overattenuated from mutations associated with passage in cultured cells [17]. To produce a live HCMV vaccine that is more immunogenic and protective than Towne vaccine, investigators previously constructed genetic recombinants in which regions from the low-passage Toledo strain of HCMV were substituted for the corresponding regions of the Towne genome.

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Correspondence: S. P. Adler, CMV Research Foundation, 514 Diane Ln, Richmond, VA 23227 (spadler123@gmail.com).

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Because it is unknown which substitutions would produce an attenuated phenotype, 4 different Towne/Toledo chimera vaccine candidates were constructed. The safety and tolerability of these 4 vaccines after administration to healthy HCMV-seropositive subjects were reported [18]. None of the 4 vaccines could be detected in blood, urine, or saliva obtained from any vaccine recipient, and there were no differences in systemic symptoms between seropositive and placebo recipients, nor were new immune responses detected to HCMV. Here we report the first evaluation of the 4 Towne/Toledo chimera vaccines in healthy HCMV-seronegative subjects.

MATERIALS AND METHODS

Study Sites

Subjects were enrolled at Virginia Commonwealth University (VCU; Richmond, Virginia).

Vaccines

Overlapping cosmid clones were prepared from genomic DNA of the HCMV Towne-varRIT and Toledo strains [19, 20]. For each strain, 8 cosmids were selected that spanned the entire HCMV genome and could regenerate infectious virus after cotransfection. The vaccine candidates (chimeras 1–4) were constructed by cotransfection of selected Towne and Toledo cosmids [19]. The Toledo unique long *UL/b'* region was in each vaccine. Because its orientation in the chimeras is opposite that in clinical HCMV isolates, this region serves as a genetic marker for the vaccines. For the clinical trial materials, DNA from the vaccine candidates was used to transfect MRC-5 cells from a characterized bank. The viruses were amplified by infection of MRC-5 cells and were harvested from the supernatants.

Subjects

Thirty-six subjects were enrolled from October 2011 through October 2014. All subjects were single males living alone, aged 22–56 years, and HCMV seronegative. The mean age was 35 years. All vaccine recipients were African American or non-Hispanic white. Persons were excluded if they were immunodeficient or had antibodies to hepatitis C virus, hepatitis B virus surface antigen, or human immunodeficiency virus (HIV). The institutional review board of VCU approved the study, and informed written consent was obtained from subjects.

Study Design

This was a phase 1 dose-escalation trial without a placebo group. The 36 subjects were enrolled into one of 4 groups, with 9 subjects per group and each group receiving one of the 4 chimera vaccines. Within each vaccine group, the first 3 subjects received 10 plaque-forming units (PFU) of the assigned vaccine; the second 3, 100 PFU; and the final 3, 1000 PFU. The vaccines were administered subcutaneously over the deltoid muscle as a single 0.5-mL dose.

Clinical Assessment

The study subjects had clinical evaluations performed weekly until 12 weeks after immunization and, for those who seroconverted to HCMV, monthly until 12 months after immunization. Clinical evaluations consisted of an interval medical history and physical examination. Subjects completed daily diary cards to describe local reactions for 4 weeks after immunization and systemic reactions for 8 weeks after immunization. Solicited local reactions included pain, erythema, induration, and tenderness. Solicited systemic symptoms included fever (oral temperature, >38°C), rash, malaise, myalgias, sore throat, headache, and nausea. The symptoms were reported as mild (causing transient or mild discomfort), moderate (causing mild-to-moderate limitation of normal daily activity), or severe (causing marked limitation of normal daily activity).

Safety Laboratories

Safety laboratory evaluations were performed monthly until 3 months after immunization and, for those who seroconverted, monthly until 12 months after immunization. These included a complete blood count (CBC) with determination of differential and quantitative platelet counts, liver function tests (to determine alanine aminotransferase [ALT], aspartate aminotransferase [AST], total bilirubin, and alkaline phosphatase levels), and measurement of creatinine levels.

Immunologic Studies

Sera and peripheral blood mononuclear cells (PBMCs) were collected weekly until 12 weeks after immunization and, for those who seroconverted, monthly until 12 months after immunization. Sera were stored at –80°C. Whole blood was collected 0, 2, 8, 12, and 24 weeks after immunization, and leukapheresis was performed at selected time points. PBMCs were harvested via Ficoll-Hypaque density-gradient centrifugation and stored in liquid nitrogen. All PBMCs were shipped to Oregon. Two enzyme immunoassays (EIAs) were used to detect seroconversion to HCMV. Immunoglobulin G (IgG) to whole viral antigen was measured using commercial reagents (Wampole Laboratories, Princeton, NJ). Detection of IgG antibodies to purified recombinant HCMV glycoprotein B (gB) was performed [21].

Neutralizing Antibody Assays

Neutralizing assays were conducted as published elsewhere [22]. Sera were diluted 1:4 and then 2-fold serially diluted in cell culture medium, incubated with an equal volume of Dulbecco's modified Eagle's medium containing 5000 PFU of the green fluorescent protein–tagged HCMV BADrUL131-Y4 for 1 hour at 37°C, and then transferred in triplicate to wells of black-walled, clear-bottomed 384-well plates containing confluent ARPE-19 or MRC-5 cells. Representative images were taken 4 or 5 days after infection, using a Nikon Diaphoto 300 fluorescence microscope. Relative fluorescent light units (RLU) were measured for each well using a Biotek Synergy HT Multi-Mode Microplate Reader 7 days after infection. Mean RLUs (from triplicate wells) were

plotted versus $\log[\text{serum dilution} - 1]$, and best-fit 4-parameter curves were determined using Prism 5 (GraphPad Software). Neutralizing titers were reported as inverse serum dilutions corresponding to 50% reductions in RLU.

Virus Detection Assays

At each visit urine and saliva were collected for HCMV culture performed using routine methods on MRC-5 cells. Cultures were monitored visually for 6 weeks for cytopathic effect.

T-Cell Assays

Frozen PBMCs from whole blood or from leukapheresis were thawed and incubated at 37°C for 12–18 hours in Roswell Park Memorial Institute medium/10% fetal bovine serum. Cells were stimulated for 6–9 hours with 2.5 µg/mL of overlapping 15-mer peptide pools from each of 20 HCMV antigens in the presence of Brefeldin A (eBioscience, 1×) and costimulation (anti-CD49d and anti-CD28 antibodies, L293 and L25 [BD Biosciences], both at 1 µg/mL or the BD-recommended concentration). Responses to overlapping peptide pools from IE1, pp65, and some or all (depending on PBMC availability) of the following HCMV antigens were assessed: IE2, UL103, UL151, UL153, UL28, UL32, UL36, UL40, UL55, UL48, UL82, UL94, UL99, US24, US29, US3, and US32 (all from JPT). Cells were stained with surface antibodies against CD3 (SK7; BD Biosciences), CD4 (SK3 [eBioscience] or OKT4 [Biolegend]), CD8 (RPA-T8; BD Biosciences), CD69 (FN50; Biolegend), CD28 (L293; BD Biosciences), CD95 (DX2; BD Biosciences), and a live cell exclusion dye (Live/Dead Fixable Aqua; Life Technologies). Cells were fixed with Cytofix/Cytoperm, permeabilized with Perm/Wash (BD Biosciences), and stained intracellularly with antibodies to tumor necrosis factor α (TNF- α ; MAb11, eBioscience) and interferon γ (IFN- γ ; 4S.B3; eBioscience). Background staining was assessed by stimulation with peptide solvent and costimulation. When surface expression of phenotypic markers was assessed, PBMCs were not stimulated with peptide but were stained immediately upon thawing. T cells were gated as live CD3⁺CD8⁺CD4⁻ cells or live CD3⁺CD8⁻CD4⁺ cells. Positive responses were defined as IFN- γ and TNF- α expression by >0.03% of total CD8⁺ or CD4⁺ T cells and >3 times the background level. Data were collected on an LSRII or Fortessa (BD Biosciences) and analyzed using FlowJo software v. 9 or 10 (TreeStar).

RESULTS

Subjects

The subjects were non-Hispanic white or African American males 20–60 years of age (Table 1). One subject who seroconverted after receiving 1000 PFU of chimera 2 was lost to follow-up after the week 44 visit.

Safety

Overall, each of the 4 Towne/Toledo chimera vaccines were safe and well tolerated. No subject had HCMV detected in either urine or saliva during the 12 weeks after immunization or 52 weeks for those who seroconverted. One subject developed mild pain without tenderness, erythema, or swelling at the site of injection, which was considered possibly related to vaccine. No subject reported systemic reactions. After immunization, both seroconverters and nonseroconverters developed transient clinically insignificant abnormal laboratory findings at the same rate (2 of 11 and 9 of 24, respectively; $P = .4$; Table 2). Abnormal laboratory findings were unrelated to vaccine dose. There were no serious adverse events associated with receipt of any vaccine.

Antibody Responses

Twenty-five subjects showed no serological evidence of infection and were not followed beyond week 12 after vaccination. The kinetics of serological responses for the 11 subjects who seroconverted are shown in Table 3. For chimera 1, only 1 of 9 subjects seroconverted (the subject received 100 PFU). For chimera 2, 3 subjects seroconverted (1 received 100 PFU, and 2 received 1000 PFU). For chimera 3, none of the 9 subjects seroconverted. For chimera 4, 7 subjects seroconverted (1 received 10 PFU, 3 received 100 PFU, and 3 received 1000 PFU). Seroconversions occurred between 4 and 12 weeks after immunization (Table 3).

Neutralizing Activity

All 11 subjects who seroconverted developed detectable levels of neutralizing activity (11 of 11, using fibroblast-based assays, and 10 of 11, using epithelial cell-based assays), as shown in Table 3. Neutralizing activity appeared on average 10 weeks after immunization (range, 4–24 weeks), slightly later than emergence of IgG against whole-virus antigens (mean, 7.4 weeks) and IgG against gB (mean, 9 weeks). Neutralizing activity also persisted longer than antibodies detected by EIAs, with neutralizing

Table 1. Demographic Characteristics of Towne/Toledo Chimera Vaccine Recipients

Characteristic	Chimera Vaccine 1 (n = 9)	Chimera Vaccine 2 (n = 9)	Chimera Vaccine 3 (n = 9)	Chimera Vaccine 4 (n = 9)	Overall (n = 36)
Age, y, mean (range)	38 (23–51)	37 (22–56)	35 (22–52)	28 (22–37)	35 (22–56)
Race/ethnicity					
African American	4	3	2	1	10
Hispanic	0	0	0	0	0
White	5	6	7	8	26

Table 2. Abnormal Laboratory Findings Among Nonseroconverters and Seroconverters

Finding	Nonseroconverters, No. of Subjects (Severity Grade)				Seroconverters, No. of Subjects (Severity Grade) ^a			
	Chimera Vaccine 1 (n = 8)	Chimera Vaccine 2 (n = 5)	Chimera Vaccine 3 (n = 9)	Chimera Vaccine 4 (n = 2)	Chimera Vaccine 1 (n = 1)	Chimera Vaccine 2 (n = 4)	Chimera Vaccine 3 (n = 0)	Chimera Vaccine 4 (n = 7)
Thrombocytopenia	0	1 (1)	0	0	0	0	0	0
Abnormal alkaline phosphatase level	0	0	0	0	0	0	0	0
Abnormal bilirubin level	0	1 (1)	0	0	0	0	0	1 (3 ^a)
Abnormal transaminase level	1 (1)	1 (1)	0	0	0	0	0	0
Atypical lymphocytosis	0	0	0	0	0	0	0	0
Anemia	1 (1)	0	0	0	0	1 (2 ^b)	0	0
Elevated WBC count	1 (1)	0	0	0	0	0	0	0
Low ANC	0	1 (2 ^c)	0	1 (2 ^c)	0	0	0	0
Total	3	4	0	2	0	1	0	1

Abbreviations: ANC, absolute neutrophil count; WBC, white blood cell.

^a Defined as >2 times the upper limit of normal.

^b Defined as 10.5–11.5 g/dL.

^c Defined as 1300–1600 cells/mm³.

activity remaining detectable for at least 8 months after being detected (Table 3). Recipients of chimera 2 or chimera 4 had similar times after immunization for first appearance of neutralizing activity or the duration of antibodies (Table 3).

Figure 1 depicts the kinetics of neutralizing activity for the 3 subjects who received 1000 PFU of chimera 4. Neutralizing activity peaked 4–12 weeks after immunization and declined to low but detectable levels by 52 weeks after immunization.

Table 3. Kinetics of Anti-Human Cytomegalovirus (HCMV) Antibody Responses in Subjects Who Seroconverted

Chimera Vaccine, Subject	Dose, PFU	Antigen Used in IgG EIA, Time First Detected (Detection Duration), wk		Cells Used in Neutralization Assay, Time First Detected (Detection Duration), wk	
		HCMV	gB	Epithelial Cells	Fibroblasts
1					
13	100	Negative	5 (>48)	20 (>4)	24 (ND)
2					
19	100	9 (5)	16 (ND)	12 (ND)	12 (>40)
28	1000	4 (20)	8 (>4)	8 (44)	8 (>32)
30	1000	9 (5)	8 (>8)	12 (>32)	12 (>32)
4					
10	10	10 (<12)	10 (>40)	11 (ND)	12 (ND)
22	100	9 (2)	11 (ND)	ND	8 (>12)
23	100	8 (<1)	8 (>4)	12 (ND)	8 (>46)
24	100	10 (22)	10 (ND)	12 (ND)	12 (>40)
34	1000	4 (5)	8 (>8)	4 (>48)	4 (>48)
35	1000	6 (2)	8 (>8)	4 (>48)	4 (>48)
36	1000	5 (5)	9 (>4)	8 (>43)	8 (>43)

Abbreviations: EIA, enzyme immunoassay; gB, HCMV glycoprotein B; IgG, immunoglobulin G; ND, not done; PFU, plaque-forming units.

Similar kinetics were observed for subjects who received chimera 2 and 4 (not shown). Figure 2 shows mean geometric mean neutralizing titers for subjects who seroconverted in response to vaccination with chimeras 2 or 4. While fibroblast entry neutralizing titers were comparable to those of naturally infected seropositive subjects, epithelial cell entry neutralizing titers were significantly lower.

T-Cell Responses

T-cell responses were assessed by intracellular cytokine staining (ICS) following stimulation of PBMCs with pools of overlapping peptides covering known immunodominant HCMV antigens (Figure 3). CD4⁺ T-cell responses were detected in 2 subjects: subject 23 responded to UL32, and subject 24 responded to pp65 and UL48 (Figure 3 and Supplementary Figure 1). CD8⁺ T-cell responses were detected in 8 of 11 seroconverters (Figure 3 and Supplementary Figure 1). All subjects in whom CD8⁺ T-cell responses could be detected responded to IE-1. In addition, 1 nonseroconverter, subject 21, mounted a CD8⁺ T-cell response to IE-1 (Supplementary Figure 2A). CD8⁺ T-cell responses to other antigens were infrequent but included responses to pp65, UL32, UL36, UL48, and UL55 in 1–2 subjects each (Figure 3 and Supplementary Figure 1). Responses were 0.03%–0.7% of total CD8⁺ T cells and peaked approximately 8 weeks after vaccination. Subsequently, these responses tended to decline slowly over time, but they remained detectable for at least 52 weeks after vaccination (Figure 3). These are de novo responses to the vaccine, not preexisting cross-reactive responses, because in the 7 subjects from whom PBMCs obtained prior to detection of HCMV vaccine-elicited T-cell responses were available, baseline T-cell responses to the IE-1 peptide pool were negligible (Figure 3). In addition, 1 subject who did not

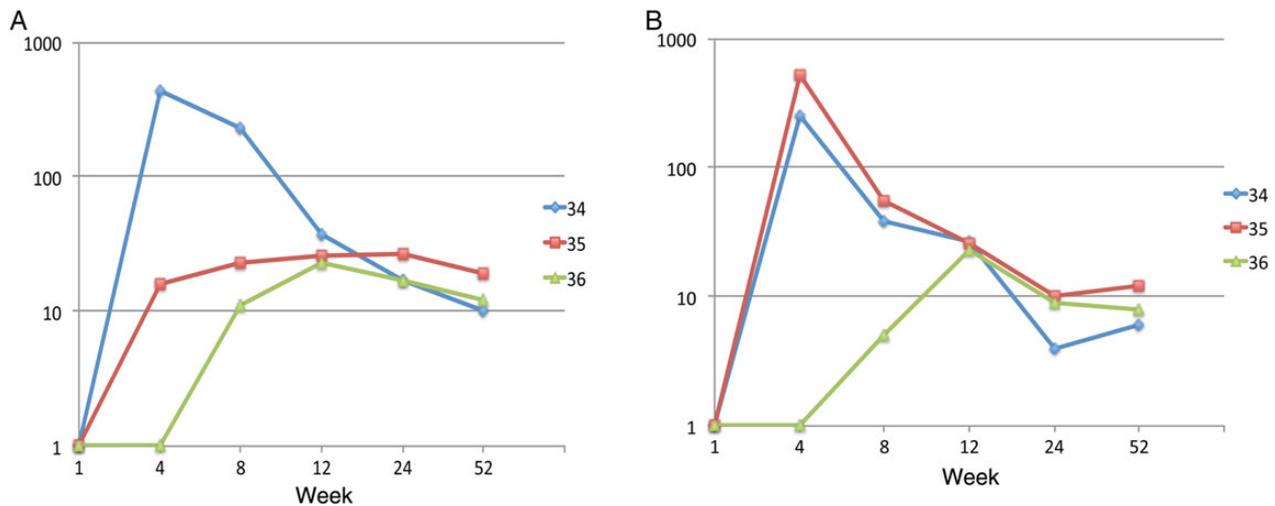


Figure 1. Neutralizing responses measured using ARPE-19 epithelial cells (A) or MRC-5 fibroblasts (B) for subjects 34, 35, and 36, who received 1000 plaque-forming units of chimera 4 vaccine.

respond to the overlapping peptide pool representing IE-1 of the AD169 laboratory strain responded to an overlapping peptide pool made using the IE-1 sequence from the vaccine strain (Supplementary Figure 2B).

Natural CMV infection causes expansion of CD8⁺ T-cell effector memory phenotype cells, sometimes to massive levels [23]. CMV vaccination in our study did not affect the proportion of total memory (CD95⁺) or effector memory (CD95⁺CD28⁻)

cells in the CD8⁺ T-cell compartment (Figure 3 and Supplementary Figure 2C). This is in contrast to infection with wild-type CMV. Subject 23 was likely exposed to wild-type CMV between week 52 and week 101 after vaccination. Note that total memory and effector memory proportions of CD8⁺ T cells remained unchanged for at least 1 year after vaccination but then dramatically expanded after a probable wild-type CMV exposure/infection, perhaps in part reflecting an anamnestic response to vaccine-induced priming (Figure 3 and Supplementary Figure 2C).

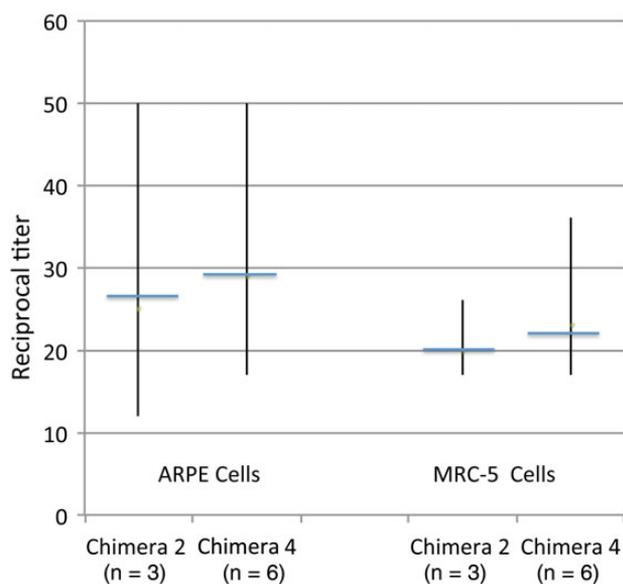


Figure 2. Geometric mean titers (GMTs) indicating neutralizing responses 12 weeks after immunization (horizontal line), with minima and maxima (solid vertical lines), among subjects who received 100 or 1000 plaque-forming units of chimera 2 or 4 vaccine. The GMTs in naturally seropositive subjects, determined using a fibroblast-based assay, is 25. The epithelial cell-based GMT for seropositive subjects is 3000.

DISCUSSION

This report describes the second human trial and the first involving HCMV-seronegative subjects of 4 genetically engineered, live HCMV vaccines. In a previous study among seropositive subjects, the same vaccines caused briefer and milder local reactions than occurred among seronegative subjects [18]. Seropositive subjects also experienced more systemic reactions than seronegative recipients.

An important objective was to determine whether chimera vaccines remained attenuated relative to the Toledo strain. A direct comparison with the Toledo strain was not possible [16, 24]. However, by comparing the clinical and laboratory safety data from the current study with earlier Toledo studies, it was possible to assess the toxicity of the chimera vaccines, compared with the Toledo vaccine.

Previously healthy HCMV-seronegative subjects who received between 10 and 100 PFU of the Toledo strain all had clinical and laboratory findings similar to those of individuals with a wild-type HCMV infection, including elevated transaminase levels, atypical lymphocytosis, and/or positive HCMV culture results [24]. Based on this previous study, we predicted that an HCMV-seronegative subject who received even 10 PFU of a

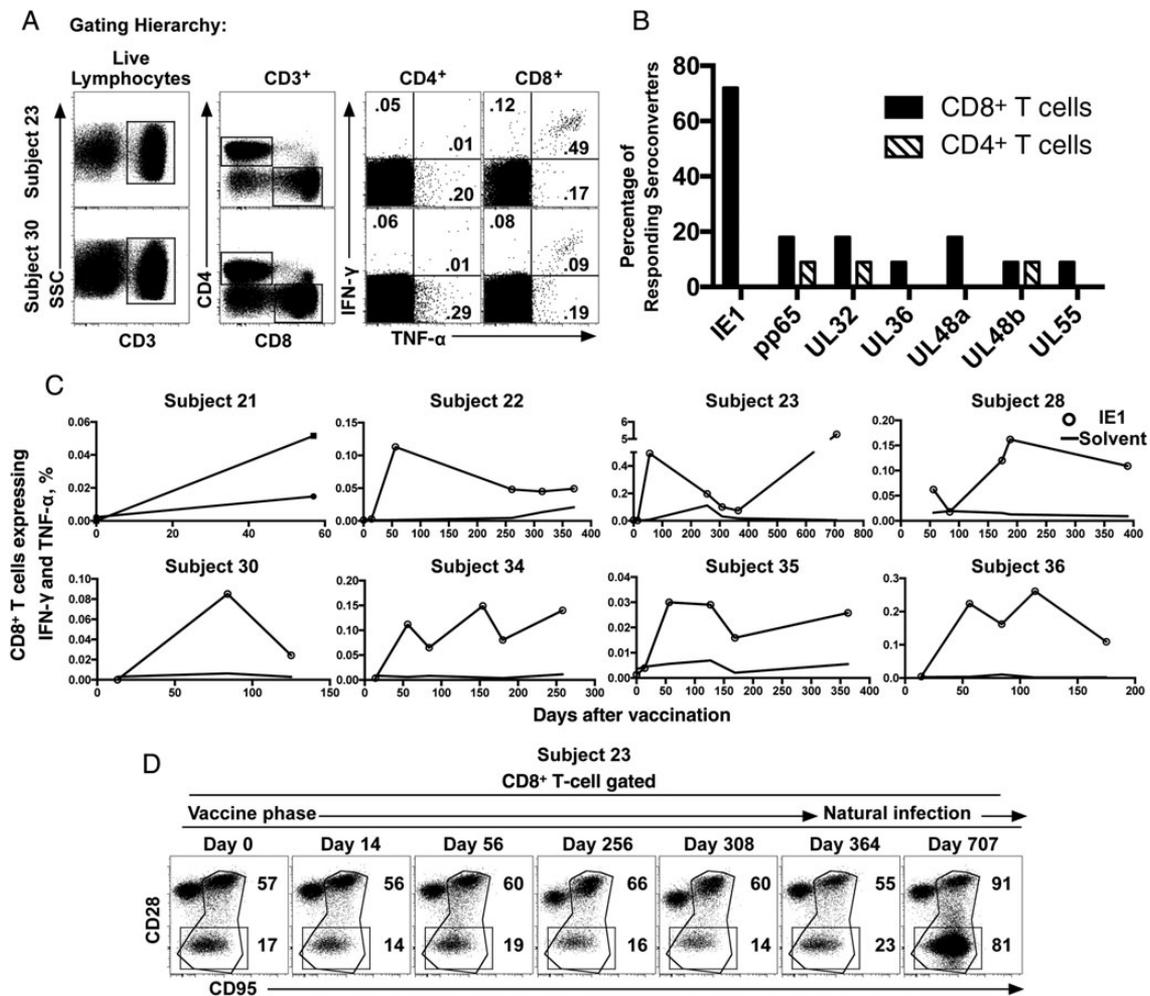


Figure 3. T-cell responses to vaccination. *A*, Gating hierarchy for assessment of T-cell responses. Shown are IE-1 responses in 2 representative subjects. *B*, Proportion of seroconverters ($n = 11$) who mounted CD4⁺ or CD8⁺ T-cell responses to overlapping peptide pools covering the indicated proteins. *C*, Kinetics of CD8⁺ T-cell responses to IE-1 overlapping peptide pools in the indicated subjects. All subjects displayed are seroconverters, except subject 21. *D*, Proportion of total memory (CD95^{+/low}) and effector memory (CD95^{+/low}CD28⁻) cells among CD8⁺ T cells over time for subject 23, who may have been exposed to wild-type human cytomegalovirus between week 52 and 101 after vaccination. Abbreviations: IFN- γ , interferon γ ; TNF- α , tumor necrosis factor α .

chimera vaccine with Toledo-like virulence would have nearly a 100% chance of developing an elevated AST level or atypical lymphocytosis or of having a positive HCMV culture result, and there would be a nearly 100% chance of observing these events in a group of 3 subjects. In the current study, however, no subject had clinical disease, and only a few subjects had abnormal laboratory findings suggestive of a Toledo-like infection. These data strongly support the conclusion that all 4 Towne/Toledo chimera vaccine candidates are attenuated.

For live HCMV vaccines of unknown safety, excretion in urine or saliva with subsequent transmission to close contacts is a concern. In previous studies, 2 of 5 naturally HCMV-seropositive subjects, 4 of 7 Towne vaccine recipients, and 6 of 6 seronegative subjects shed Toledo strain following experimental challenge with Toledo [16,24]. In the current study, we detected no infectious virus in urine or saliva samples of vaccine

recipients. This suggests that, like the Towne vaccine, the Towne/Toledo chimera vaccines do not establish systemic infection, and they further support the conclusion that they are greatly attenuated. Given the extreme sensitivity of polymerase chain reaction (PCR), we did not test specimens by PCR for chimera DNA, which, if positive, may not have reflected infectious virus, but rather only whole or partial genomes.

Regarding immunologic responses among vaccines, differences were clearly apparent, with chimera 4 clearly the most successful at inducing seroconversion. However, among all who seroconverted, the magnitude and duration of the serologic responses, including neutralizing titers, measured using both fibroblast- and epithelial cell-based assays, were similar to those previously observed for Towne vaccine [25] and were not notably more rapid or more robust for some Chimera vaccines over others.

CD8⁺ T-cell responses to IE-1 were detected in after seroconversion in most subjects and in 1 subject who did not seroconvert. The magnitude of the responses was a little lower than previously seen in the Towne vaccine study. Furthermore, CD4⁺ T-cell responses were detected both by proliferation and ICS in that study, whereas they were undetectable by ICS for most subjects in this study. ICS assays are subject to variability from laboratory to laboratory in reporting the magnitude of detected responses, and this could contribute to the differences seen with the previous study [26]. Nevertheless, these results suggest that chimeras 2 and 4 were used at suboptimal doses for eliciting cellular responses. The optimal dose of Towne vaccine is 3000 PFU [27].

The Toledo *UL/b'* region was in each vaccine. Its unique orientation distinguishes the vaccine viruses from wild-type virus. A rationale for including this region was that genetic data indicated that 16 *UL/b'* genes (*UL133* to *UL150A*) were missing from Towne. Thus, adding this region may have increased the immunogenicity of the vaccines relative to Towne because the missing genes encode targets of humoral immune responses [17, 28, 29] and proteins that influence HCMV cellular tropism and host responses to infection [20, 27, 30–33]. Subsequently, we found that only half of the Towne virions have this deletion. The other half, called Towne-varL, contain a wild-type *UL/b'* region. Thus, neither the attenuation of Towne or its variable immunogenicity or efficacy are due to absence of these genes.

Genomic sequencing identified 5 mutations in Towne-varL that disrupt expression of proteins encoded by annotated genes. These mutations, located in genes *RL13*, *UL1*, *UL40*, *UL130*, and *US1*, were presumably acquired during in vitro passage and are therefore implicated in attenuation of Towne. *UL130* is a subunit of the pentameric complex, an envelope glycoprotein complex comprising gH, gL, *UL128*, *UL130*, and *UL131A* [34]. The *UL128*, *UL130*, and *UL131A* subunits are dispensable for fibroblast entry, but all 3 are necessary for HCMV entry into epithelial, endothelial, and myeloid lineage cells. Thus, failure to express *UL130* may attenuate Towne by limiting cells available for viral replication and dissemination in vivo to those of fibroblast/mesenchymal lineage. Conversely, the pentameric complex is a target for antibodies that neutralize epithelial cell entry, and Towne's efficacy may be due to its inability to induce epithelial entry-specific neutralizing antibody responses.

The Toledo genome contains mutations disrupting *RL13*, *UL9*, *UL36*, and *UL128*, and a 14-kb inversion of *UL/b'* sequences encoding *UL130* to *UL148*. Thus, owing to disruption of *UL128*, Toledo resembles Towne by not expressing the pentameric complex and consequently lacks epithelial/endothelial cell tropism. However, as human studies used Toledo passage 4 or 5 and Toledo genomic sequence data are from passage 8, it is possible that these mutations occurred during subsequent passage. Unfortunately, samples of Toledo prior to passage 8 are unavailable.

The pentameric complex in the 4 Towne/Toledo chimera vaccines is of interest. The Toledo *UL/b'* region, included in all 4 chimeras, contains wild-type *UL130* and *UL131A*, while according to available genome maps Towne *UL* sequences in chimeras 1 and 2 contain wild-type *UL128*. Thus, chimeras 1 and 2 appear to contain the open reading frames necessary to express and assemble a functional pentameric complex. Nevertheless, all 4 chimeras lacked epithelial tropism in cell culture (data not shown). This is consistent with in vivo results that all 4 chimeras are no more virulent nor immunogenic (with respect to epithelial entry neutralizing responses) than Towne vaccine. Thus, concerns that the Towne/Toledo chimera vaccines may have epithelial tropism appear unfounded. There was no evidence that viral replication occurred in vivo for any chimera.

The genome sequences for 4 Towne/Toledo chimeras have been determined. Each chimera contained disruptive mutations in *RL13* derived from either Towne or Toledo, and each contained a disrupted *UL128* derived from Toledo. Thus, like Towne and Toledo, the chimeras are predicted to lack epithelial/endothelial cell tropism and to be incapable of inducing pentameric complex-specific antibodies. Consistent with this, the chimeras all failed to infect epithelial cells in culture (data not shown) and in vivo are no more virulent nor immunogenic (with respect to epithelial cell entry neutralizing responses) than Towne vaccine. With respect to other gene-disruptive mutations unique to each chimera, no clear associations emerged to account for success (chimera 4) or failure (chimera 3) in establishing immunogenicity. A report of the genetics of each chimera is planned.

In summary, the present study demonstrated that Towne/Toledo chimera vaccine candidates are well tolerated in HCMV-seronegative subjects and are greatly attenuated relative to the Toledo strain or to wild-type HCMV. Chimera 4 was the most immunogenic. On the basis of these results, future studies are warranted to determine the safety and immunogenicity of these vaccines in additional subjects and at higher doses to establish the optimal dosages of these vaccines.

Supplementary Data

Supplementary materials are available at <http://jid.oxfordjournals.org>. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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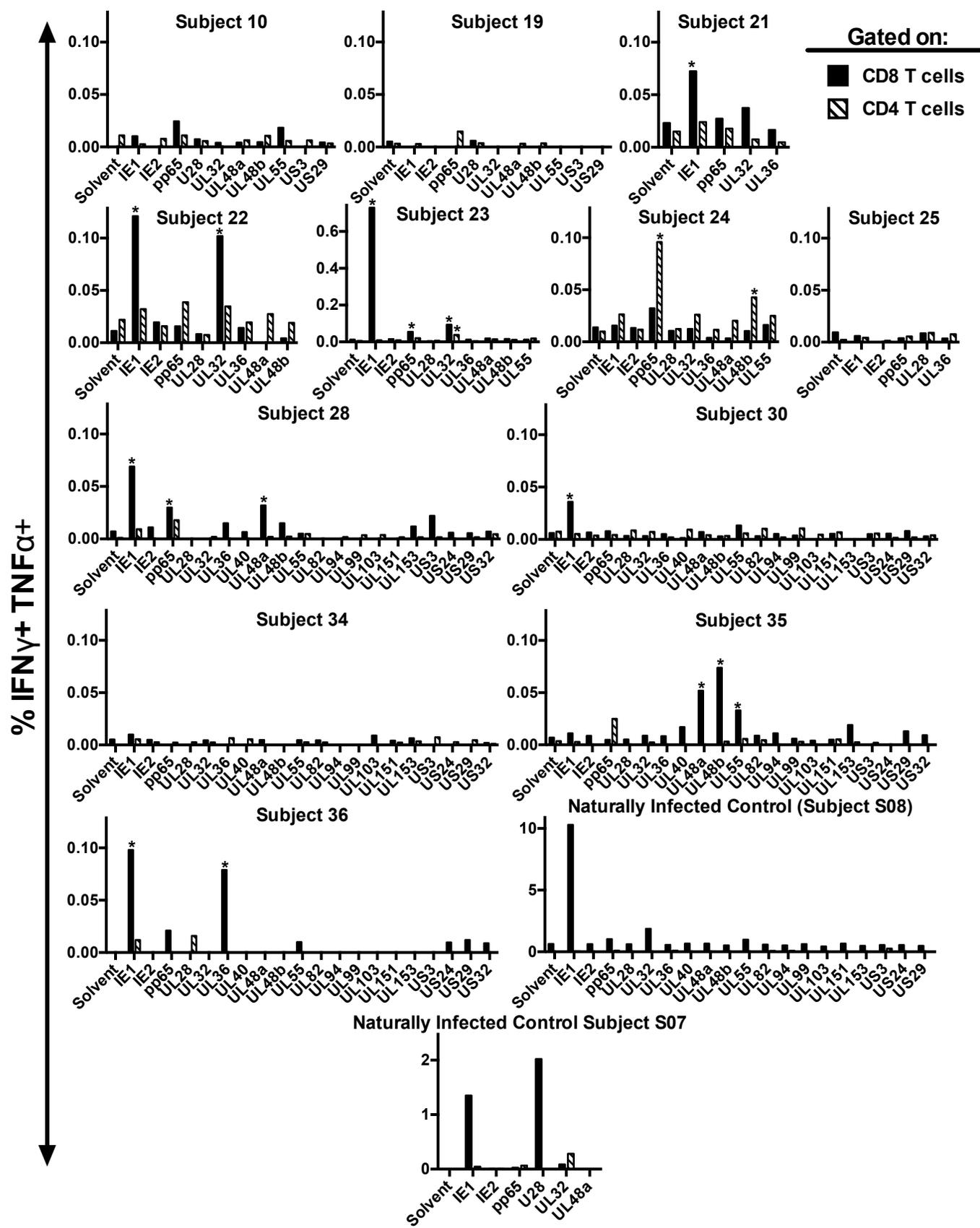
International Development and other donors (a full list of IAVI donors is available at: <http://www.iavi.org>).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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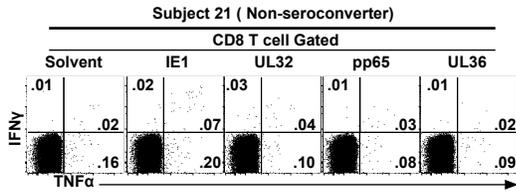
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Supplementary Figure 1

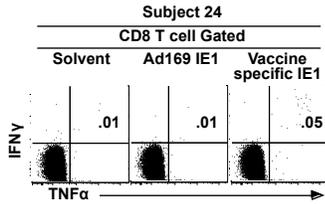


Supplementary figure 2

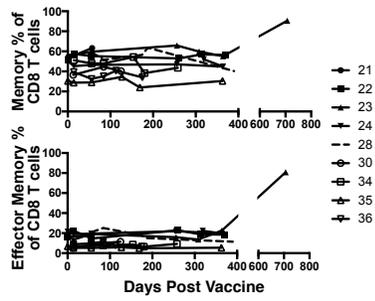
A



B



C



Supplementary Figure 1. Responses to all tested overlapping peptide pools. Responses in all seroconverters and in two naturally infected controls are shown. Non-seroconverters in the 100pfu and 1000pfu doses were also tested for IE-1 responses. Subject 21 was the only non-seroconverter who mounted an IE-1 CD8 T cell response; all other non-seroconverters not shown. *Subject responses considered positive ($>0.03\%$ and $>3x$ over background); data summarized in Figure 1B.

Supplementary Figure 2. Additional CD8 T cell responses and phenotypes. A, CD8 T cell responses in non-seroconverter, subject 21. B, Vaccine-specific IE-1 response in subject 24. C, Proportion of total memory (CD95⁺/low) and effector memory (CD95⁺/lowCD28⁻) within CD8 T cells over time for all subjects who mounted a vaccine-specific CD8 T cell response.