MAJOR ARTICLE

# Safety and Immunogenicity of a Bivalent Cytomegalovirus DNA Vaccine in Healthy Adult Subjects

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**(See the editorial commentary by Go and Pollard, on pages 1631–3.)**

***Background.*** VCL-CB01, a candidate cytomegalovirus (CMV) DNA vaccine that contains plasmids encoding CMV phosphoprotein 65 (pp65) and glycoprotein B (gB) to induce cellular and humoral immune responses and that is formulated with poloxamer CRL1005 and benzalkonium chloride to enhance immune responses, was evaluated in a phase 1 clinical trial.

***Methods.*** VCL-CB01 was evaluated in 44 healthy adult subjects (22 CMV seronegative and 22 CMV seropositive) 18–43 years old. Thirty-two subjects received 1- or 5-mg doses of vaccine on a 0-, 2-, and 8-week schedule, and 12 subjects received 5-mg doses of vaccine on a 0-, 3-, 7-, and 28-day schedule.

***Results.*** Overall, the vaccine was well tolerated, with no serious adverse events. Local reactions included mild to moderate injection site pain and tenderness, induration, and erythema. Systemic reactions included mild to moderate malaise and myalgia. All reactions resolved without sequelae. Through week 16 of the study, immunogenicity, as measured by enzyme-linked immunosorbant assay and/or ex vivo interferon (IFN)–$\gamma$ enzyme-linked immunospot assay, was documented in 45.5% of CMV-seronegative subjects and in 25.0% of CMV-seropositive subjects who received the full vaccine series, and 68.1% of CMV-seronegative subjects had memory IFN-$\gamma$ T cell responses at week 32.

***Conclusion.*** The safety and immunogenicity data from this trial support further evaluation of VCL-CB01.

Cytomegalovirus (CMV), a betaherpesvirus, infects 50%–85% of adults by 40 years of age [1]. Most healthy individuals who acquire CMV after birth develop few, if any, symptoms; however, CMV disease causes significant morbidity and mortality in immunocompromised individuals, such as recipients of hematopoietic cell transplants (HCT) and solid-organ transplants [2]. In HIV-infected individuals, CMV infection accelerates progression to AIDS and death, despite antiretroviral therapy [3]. In the United States, congenital abnormalities due to transplacental infection with CMV lead to death or birth defects, including deafness and mental retardation, in ~8000 infants each year [4, 5]. A CMV vaccine is currently not available, even though the Institute of Medicine ranked CMV as the top priority for vaccine development in the United States [6].

The incidence of CMV antigenemia in CMV-seropositive HCT recipients who do not receive prophy-

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**Table 1. Subject distribution.**

| Group | Injection schedule | Dose per injection, mg | CMV-seropositive subjects | CMV-seronegative subjects |
|---|---|---|---|---|
| 1 | Week 0, 2, 8 | 1 | 8[a] | 8[b] |
| 2 | Week 0, 2, 8 | 5 | 8[c] | 8 |
| 3 | Day 0, 3, 7, 28 | 5 | 6 | 6 |
| Total | | | 22 | 22 |

**NOTE.** CMV, cytomegalovirus.

[a] One subject, who received only a single injection, discontinued the trial after week 2; a second subject, who received all 3 injections, discontinued the trial after week 10.

[b] One subject, who received all 3 injections, discontinued the trial after week 24.

[c] One subject, who received only 2 injections, discontinued the trial after week 2.

laxis is 50%–70% during the first 100 days after transplant [7, 8]. Preemptive antiviral therapy reduces the incidence of CMV-associated disease to ~5% [9]; however, drug toxicity, the expense of antiviral treatment, and the possibility of the emergence of drug-resistant viruses are major drawbacks to the use of antivirals for prevention of CMV disease. Even with antiviral therapy, patients may develop viremia, or they may develop "late-onset" CMV viremia and disease after the therapy is discontinued [10, 11]. A CMV vaccine that enables the patient's immune system to control CMV infection, resulting in a reduced need for antiviral therapy, would be a valuable therapeutic option for HCT recipients.

Control of CMV in immunocompromised persons is primarily associated with cellular immune responses. Both $CD8^+$ and $CD4^+$ T cells appear to be important for protection against CMV disease [12, 13]. A recent study of CMV-specific $CD4^+$ and $CD8^+$ T cells from healthy donors used overlapping peptides from 213 CMV open reading frames to identify antigens recognized after CMV infection [14]. The CMV tegument phosphoprotein 65 (pp65) and the major CMV surface glycoprotein B (gB) were the antigens most frequently recognized by $CD4^+$ T cells, and pp65 was also one of the antigens most frequently recognized by $CD8^+$ T cells.

The development of a vaccine for the prevention of congenital infection by transplacental transmission of CMV is also a high priority. In contrast to the transplant setting, antibodies to surface glycoproteins, especially gB, appear to be critical for protection against the maternal-fetal transfer of CMV [15]. In addition, CMV-specific T cell responses are also likely to play an important role in reducing viral load in the mother and, thus, exposure of the fetus.

A CMV vaccine that induces protective T cell and antibody responses has the potential to prevent infection or ameliorate CMV disease due to congenital infection or transplantation. To that end, we developed a CMV DNA vaccine, VCL-CB01, composed of two plasmids with human codon-optimized CMV genes, pp65 and gB [16]. CMV pp65 was included to induce T cell responses; gB was included to induce antibodies and T cell responses. VCL-CB01 was formulated with poloxamer CRL1005 and benzalkonium chloride (BAK) to increase immunogenicity.

## MATERIALS AND METHODS

***VCL-CB01 vaccine.*** VCL-CB01, a bivalent CMV DNA vaccine consisting of 2 plasmids, VCL-6368 and VCL-6365, formulated with poloxamer CRL1005 and BAK in PBS, has been described elsewhere [16]. VCL-6368 encodes pp65 from AD169 with the putative protein kinase domain removed by deletion of aa 435–438. VCL-6365 encodes the extracellular domain (aa 1–713) of CMV gB. Formulation of the 2 plasmids with CRL1005 and BAK produces a thermodynamically stable, self-assembled particulate system with a defined particle size, surface charge, and stability profile.

***Trial design.*** VCL-CB01 was evaluated for safety and immunogenicity in a phase 1, multicenter, open-label, dose-escalating trial in healthy CMV-seropositive and CMV-seronegative adults. Subjects received intramuscular (deltoid) injections of 1- or 5-mg doses of VCL-CB01 on a 0-, 2-, and 8-week schedule (groups 1 and 2, respectively) or 5-mg doses of VCL-CB01 on a 0-, 3-, 7-, and 28-day schedule (group 3). Blood was collected for assessment of immune responses at baseline and weeks 2, 4, 8, 10, 16, 24, and 32 for groups 1 and 2 and at baseline and weeks 1, 2, 4, 6, 12, and 16 for group 3. The end points of the trial were safety and immunogenicity, as defined by gB antibody responses measured by ELISA and pp65 interferon (IFN)–$\gamma$ T cell responses measured by ex vivo enzyme-linked immunospot (ELISPOT) assay. An additional assay, the cultured IFN-$\gamma$ ELISPOT assay, was used to further evaluate immunogenicity.

An institutional review board approved the clinical protocol and informed consent at each of 4 sites, and written informed consent was obtained at enrollment from each volunteer before any procedures were conducted.

***Safety assessment.*** Safety was assessed by measurement of vital signs, laboratory tests, review of reactogenicity 30 min after each injection, symptom-directed clinical evaluations, postinjection subject diaries, adverse event (AE) monitoring, and re-

**Table 2. Maximum toxicity grade (MTG) of related adverse events (AEs), by treatment group.**

| | Group 1 MTG ($n$ = 16) | | | Group 2 MTG ($n$ = 16) | | | Group 3 MTG ($n$ = 12) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| AE | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | Total[a] |
| General disorders and administration site conditions | | | | | | | | | | |
| Injection site pain | 7 | 2 | 1 | 10 | 4 | 0 | 4 | 8 | 0 | 36 (81.8) |
| Malaise | 4 | 1 | 0 | 5 | 1 | 0 | 5 | 2 | 0 | 18 (40.9) |
| Feeling hot and cold | 1 | 1 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 5 (11.4) |
| Injection site induration | 0 | 0 | 0 | 3 | 0 | 0 | 2 | 0 | 0 | 5 (11.4) |
| Fatigue | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 (6.8) |
| Injection site erythema | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 3 (6.8) |
| Injection site pruritis | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 3 (6.8) |
| Pyrexia | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 3 (6.8) |
| Feeling hot | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 (4.5) |
| Injection site swelling | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 (4.5) |
| Rigors | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 2 (4.5) |
| Musculoskeletal and connective tissue | | | | | | | | | | |
| Myalgia | 3 | 3 | 0 | 8 | 3 | 0 | 6 | 1 | 0 | 24 (54.5) |
| Nervous system | | | | | | | | | | |
| Headache | 5 | 1 | 0 | 7 | 0 | 0 | 5 | 0 | 0 | 18 (40.9) |
| Gastrointestinal | | | | | | | | | | |
| Nausea | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 3 (6.8) |
| Skin and subcutaneous tissue disorders | | | | | | | | | | |
| Erythema | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 (4.5) |
| Pruritis | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 (4.5) |
| Rash | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 2 (4.5) |
| Investigations | | | | | | | | | | |
| Decrease in Hb level | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 (4.5) |

**NOTE.** No toxicity grade 4–related AEs were reported. Subjects may have had >1 occurrence of the same event; however, only 1 occurrence was counted per subject. Hb, hemoglobin.

[a] Data are the no. (%) of subjects with a related AE from among the total no. of subjects ($n$ = 44).

view of concomitant medication usage. Toxicity tables and grades established by the National Institute of Allergy and Infectious Diseases were used for evaluating AEs.

***Ex vivo ELISPOT assay.*** Peripheral blood mononuclear cells (PBMCs) isolated from blood shipped overnight from the study sites were cryopreserved after each blood collection. The ex vivo ELISPOT assay for detection of IFN-$\gamma$–secreting T cells was developed and qualified at the Vical Clinical Immunoassay Laboratory. The assay was performed with thawed PBMCs obtained at multiple time points, including baseline, in the same plate. In our experience, cryopreservation of PBMCs for <1 year does not reduce responses relative to fresh PBMCs in the ex vivo ELISPOT assay (authors' unpublished data); moreover, the use of cryopreserved PBMCs allows batching of PBMCs from various time points in a single assay to minimize the effects of assay variability.

For the ex vivo ELISPOT assay, PBMCs at 200,000 cells/well in 96-well plates were stimulated overnight at 37°C in 5% $CO_2$ with pools of overlapping 15mer peptides (Biosynthesis). CMV pp65 and gB peptides, derived from sequences of antigens encoded in VCL-CB01, either were in a single pool of 137 or 176 peptides, respectively, at 7.5 $\mu$g/mL for each peptide or were split into 2 pools at 10 $\mu$g/mL for each peptide. Wells were coated with anti–human IFN-$\gamma$ antibody (BD Pharmingen) and were developed by sequential addition of biotinylated anti–human IFN-$\gamma$ antibody (BD Pharmingen), avidin-horseradish peroxidase (HRP) (Vector Laboratories), and 3-amino-9-ethylcarbazole (AEC) substrate (BD Pharmingen). Spot-forming units were counted with an ImmunoSpot Analyzer (CTL). Results were expressed as the number of spot-forming units per $1 \times 10^6$ PBMCs after subtraction of the number of spot-forming units in wells without peptides. Controls included phytohemagglutinin (PHA)–stimulated PBMCs, wells without peptide, a pool of 98 overlapping peptides derived from CMV immediate-early antigen 1 (IE1) (which is not encoded in VCL-CB01), and control PBMCs with established ranges for pp65, IE1, and gB.

***Cultured ELISPOT assay.*** PBMCs were seeded at $2 \times 10^6$ cells/well in 24-well plates and cultured for 10 days at 37°C in 5%

**Table 3. Vaccine responders, by group and cytomegalovirus (CMV) serostatus.**

| | Group 1 | | Group 2 | | Group 3 | |
|---|---|---|---|---|---|---|
| Response | SN ($n$ = 8) | SP ($n$ = 8) | SN ($n$ = 8) | SP ($n$ = 8) | SN ($n$ = 6) | SP ($n$ = 6) |
| gB antibody | 2 (25.0) | 0 (0) | 2 (25.0) | 0 (0) | 1 (16.7) | 0 (0) |
| pp65 ex vivo IFN-γ ELISPOT | 2 (25.0) | 3 (37.5) | 3 (37.5) | 1 (12.5) | 3 (50.0) | 1 (16.7) |
| gB ex vivo IFN-γ ELISPOT | ND | ND | 2 (25.0) | ND | 2 (33.3) | ND |
| Total | 3 (37.5) | 3 (37.5) | 4 (50.0) | 1 (12.5) | 3 (50.0) | 1 (16.7) |

**NOTE.** Data are no. (%) of subjects. Vaccine responders were defined as those with a vaccine-induced antibody or T cell response before or at week 16 (day 112). Antibody responders to the CMV glycoprotein B (gB) were defined as those who had serum specimens that were negative by the gB ELISA before vaccination and that were positive by the assay after vaccination or those who had serum specimens that were positive by the assay at baseline and had a postvaccination increase in gB antibody level of ⩾4-fold relative to baseline. Ex vivo interferon (IFN)–γ enzyme-linked immunospot (ELISPOT) assay responders to the CMV phosphoprotein 65 (pp65) or gB were defined as those with negative results by the ex vivo IFN-γ ELISPOT assay before vaccination and positive results by the assay after vaccination or those with positive results by the ex vivo IFN-γ ELISPOT assay before vaccination and a postvaccination increase in the level of pp65-specific IFN-γ–secreting T cells of >2.4-fold. ND, not done; SN, seronegative; SP, seropositive.

$CO_2$ with overlapping peptides (described above) at 0.2 μg/mL for each peptide. Separate wells were cultured with pp65, gB, or IE1 peptides. Recombinant human interleukin (IL)–2 (eBioscience) at 900 U/mL was added on days 3 and 7. After 10 days, cells were washed and rested overnight in medium without peptides. The IFN-γ ELISPOT assay was performed as described above except that cells were plated at 40,000 cells/well. Data were expressed as the number of spot-forming units per $1 \times 10^6$ cultured cells after subtraction of the number of spot-forming units in wells without peptide. Controls included wells without peptide, wells with IE1 peptides, and control PBMCs cultured in the same manner as the test samples.

***CMV gB antibody ELISA.*** Serum gB-specific IgG antibodies were detected in a gB-binding ELISA developed and qualified at the Vical Clinical Immunoassay Laboratory. The assay uses 96-well plates coated with 2 μg/mL recombinant gB purified from stably transfected CHO cells (gift from Sanofi-Aventis). Serum specimens were screened for gB binding at 1:100 and, if positive, serial dilutions of the serum were assayed with a reference serum to determine anti-gB antibody levels in ELISA units (EU) per milliliter. Antibody binding was detected with an HRP-conjugated goat anti–human IgG Fc antibody (Jackson ImmunoResearch), followed by ABTS substrate (KPL) and stop solution (KPL). Absorbance was read at 405 nm with a reference at 490 nm, and gB antibody levels were interpolated from a standard curve. Controls in the assay included positive and negative serum specimens with established ranges for EU per milliliter and reagent control wells.

***Statistical analyses.*** The primary analysis was the incidence of AEs. Descriptive summary statistics for continuous variables, including the mean, SD, median, minimum, and maximum, were used to summarize safety data. Summaries for categorical variables were presented as counts and percentages. Fisher's exact test was used to test for significant group differences.

## RESULTS

***Trial subjects.*** A total of 44 subjects (22 CMV seropositive and 22 CMV seronegative) were enrolled, including 18 men (40.9%) and 26 women (59.1%) (table 1). The race characteristics of the subject population were as follows: 37 white (84.1%), 3 African American (6.8%), 1 Asian (2.3%), and 3 other/unknown (6.8%). Seven subjects (15.9%) were Hispanic or Latino, and 37 (84.1%) were not. The mean age of the subjects was 28.6 years and ranged from 18 to 43 years. Four subjects discontinued the study; 2 discontinued before receiving the third dose of vaccine, and 2 discontinued after receiving all of the vaccine doses. No subject discontinued due to vaccine-related AEs.

***Safety.*** In general, VCL-CB01 was well tolerated. No serious AEs were reported. The most common vaccine-related AEs consisted of injection site pain (81.8%), myalgia (54.5%), headache (40.9%), and malaise (40.9%). AEs were generally of mild to moderate severity. Thirty-six subjects (81.8%) experienced at least 1 related grade 1 AE. Eighteen subjects (40.9%) developed at least 1 related grade 2 AE. One related grade 3 AE (pain at the injection site) was experienced by 1 subject after the second 1-mg injection (day 14), which abated to a grade 2 level within 1 day and resolved within 10 days. There were no grade 4 AEs. The maximum toxicity grades of related AEs, sorted by treatment group, are shown in table 2. The duration of the most commonly reported related AEs ranged from 1 to 4 days, with the exception of 1 grade 2 AE (feeling hot or cold) in 1 subject, with a duration of 7 days.

Group 2 and 3 subjects generally experienced more local reactogenicity than did group 1 subjects. In particular, there was more injection site pain with the higher dose (group 2, 87.5%) and accelerated schedule (group 3, 100%) versus group 1 (62.5%). Similarly, only subjects in groups 2 and 3 experienced injection site erythema (6.2% and 16.7%, respectively) and injection site induration (18.8% and 16.7%, respectively). Injection site swelling was reported for subjects in group 3 only

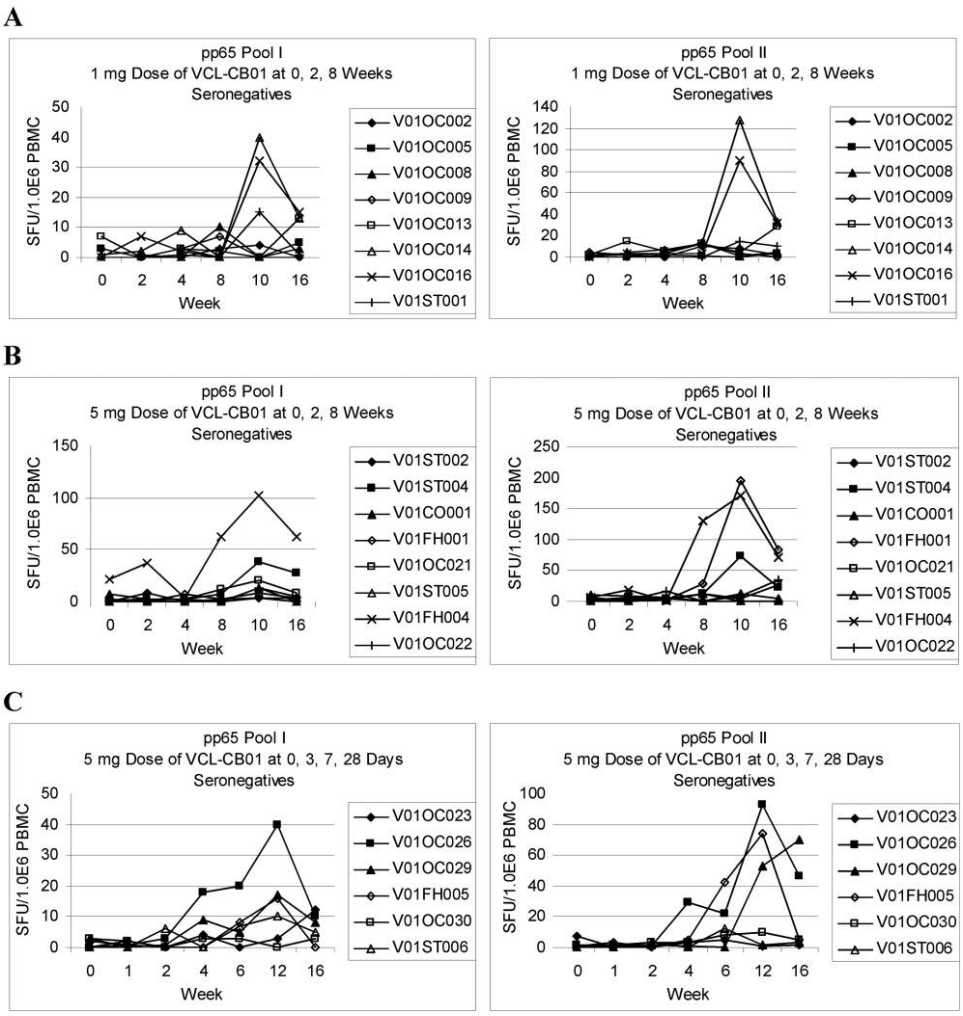


**Figure 1.** T cell responses for cytomegalovirus (CMV)–seronegative subjects. Ex vivo interferon (IFN)–$\gamma$ enzyme-linked immunospot (ELISPOT) assays were performed as described in Materials and Methods. Individual CMV phosphoprotein 65 (pp65) T cell responses are shown for CMV-seronegative subjects vaccinated with 1-mg *(A)* or 5-mg *(B)* doses of VCL-CB01 at 0, 2, and 8 weeks and for CMV-seronegative subjects with 5-mg doses of VCL-CB01 at 0, 3, 7, and 28 days *(C)*. ELISPOT results are shown as the no. of spot-forming units per $1 \times 10^6$ peripheral blood mononuclear cells (PBMCs).

(16.7%). Dose and injection schedule had no consistent relationship to the frequency of systemic symptoms. In addition, the onset of the immunogenic response did not correlate with an increase in the number of local or systemic AEs.

***Immunogenicity.*** The immunogenicity of VCL-CB01 was initially assessed through week 16 by the pp65 ex vivo ELISPOT assay for T cell responses and at all time points by gB-binding ELISA for antibody responses. In addition, gB T cell responses were assessed for CMV-seronegative subjects in group 2 at week 10 and in group 3 at week 12 by the IFN-$\gamma$ ELISPOT assay. As indicated in table 3, CMV-seronegative subjects in all groups had vaccine-induced pp65 and/or gB T cell responses (25.0%–50.0%) and gB antibody responses (16.7%–25.0%). The gB antibody responses detected with the gB ELISA were not likely to have been induced as a result of CMV infection during the course of the trial, because all CMV-seronegative subjects were negative by CMV ELISA (Diamedix CMV IgG and IgM ELISA) at the last time point in the trial (authors' unpublished data). CMV-seropositive subjects in all groups had vaccine-induced increases in pp65-specific T cells (range, 12.5%–37.5%), but, for CMV-seropositive subjects in any group, gB antibody levels did not increase >2-fold over baseline after vaccination.

***Immune response kinetics.*** Individual results and time courses for T cell and antibody responses through week 16 for CMV-seronegative subjects are shown in figures 1 and 2. In general, relative to the initial injection, T cell responses peaked by weeks 10–12 and antibody responses peaked by weeks 12–16. The kinetics of the responses relative to the first injection appear to be similar for both injection schedules.

***Priming of memory T cells.*** To more fully evaluate the ability of VCL-CB01 to prime for memory T cell responses, we assayed PBMCs from week 32 for CMV-seronegative subjects in groups 1 and 2 by the ex vivo ELISPOT assay. In addition, PBMCs from week 32 for CMV-seronegative subjects in groups

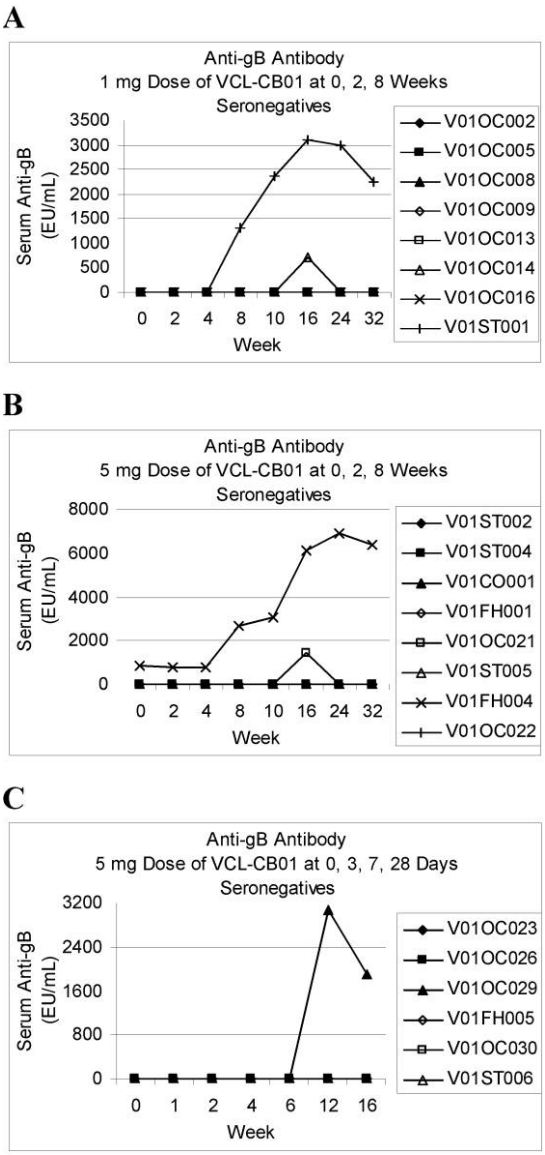


**Figure 2.** Antibody responses for cytomegalovirus (CMV)–seronegative subjects. CMV glycoprotein B (gB) ELISAs were performed as described in Materials and Methods. A serum specimen was considered to be positive if the absorbance for serum at a 1:100 dilution was greater than a reactivity threshold calculated as 2.5 times the absorbance of pooled CMV-seronegative serum specimens at a 1:100 dilution. Anti-gB levels (shown in ELISA units [EU] per milliliter) were interpolated from a standard curve using serum with well-defined reactivity to CMV gB. In a survey of 76 CMV-seronegative serum specimens and 55 CMV-seropositive serum specimens, the assay had a sensitivity of 100% and specificity of 95%. The results for the CMV-seropositive serum specimens ranged from 16,350 to 413,440 EU/mL, with a median value of 154,560 EU/mL (authors' unpublished data). Individual gB antibody responses are shown for CMV-seronegative subjects vaccinated with 1-mg *(A)* or 5-mg *(B)* doses of VCL-CB01 at 0, 2, and 8 weeks and for CMV-seronegative subjects vaccinated with 5-mg doses of VCL-CB01 at 0, 3, 7, and 28 days *(C)*.

1 and 2 and from week 16 for group 3 were evaluated by the cultured ELISPOT assay, which may be more sensitive for assessing DNA vaccine priming of memory responses. Distinct from the ex-vivo ELISPOT assay, in which PBMCs are cultured overnight with peptides, the cultured ELISPOT assay involves culturing PBMCs with peptides and recombinant human IL-2 for 10 days before use in the IFN-$\gamma$ ELISPOT assay. Thus, the cultured ELISPOT assay detects antigen-specific T cells with the capacity to proliferate and secrete IFN-$\gamma$ on restimulation with antigen [17].

As indicated in table 4, antigen-specific pp65 or gB IFN-$\gamma$ T cell responses were detected by ex vivo and/or cultured ELISPOT assay in 15 (68.1%) of 22 CMV-seronegative subjects up to 24 weeks after the last injection. No memory T cell responses were detected after culture with the IE1-specificity control peptides and IL-2.

Group 1 responders who were initially identified by the ex vivo ELISPOT assay at earlier time points failed to demonstrate responses by the same ex vivo assay by week 32. All subjects in group 2 who had T cell responses by week 16 by the ex vivo ELISPOT assay also had detectable responses by the same assay at week 32. Interestingly, 2 additional subjects in group 2 (V01OC021 and V01OC022) had detectable pp65 and/or gB responses by the ex vivo ELISPOT assay at week 32 even though T cell responses had not been previously detected in PBMCs from those subjects through week 16. Two of 3 responders in group 3 had a detectable pp65 T cell responses by the ex vivo ELISPOT assay at week 16.

DNA vaccine–induced memory T cell responses were detected by the cultured ELISPOT assay in PBMCs from 6 subjects in group 1 (75.0%), 5 subjects in group 2 (62.5%), and 4 subjects in group 3 (66.7%). Furthermore, memory T cell responses were detected in 5 subjects (4 in group 1 and 1 in group 3) who failed to demonstrate a T cell response by the ex vivo ELISPOT assay at any time point. Thus, the cultured ELISPOT assay appears to be more sensitive than the ex-vivo ELISPOT assay for detection of vaccine-induced antigen-specific T cell responses.

## DISCUSSION

The results from this trial show that VCL-CB01, a bivalent CMV DNA vaccine, was generally well tolerated and that the severity of AEs did not increase significantly with increasing dose or with an accelerated vaccination schedule. Through week 16 of the study, immunogenicity, as measured by ELISA and/or ex vivo ELISPOT assay, was documented in 45.5% of CMV-seronegative subjects and 25.0% of CMV-seropositive subjects who received the full vaccine series. Two additional subjects had detectable IFN-$\gamma$ T cell responses only at week 32, and 68.1% of subjects had memory IFN-$\gamma$ T cell responses. In CMV-seropositive subjects, VCL-CB01 boosted existing pp65 T cell responses but not gB antibody responses, possibly because of the high baseline levels of gB antibody in subjects with chronic CMV infection.

**Table 4. Ex vivo and cultured interferon (IFN)–γ enzyme-linked immunospot (ELISPOT) assay responses for cytomegalovirus (CMV)–seronegative subjects.**

| Group, subject | Ex vivo ELISPOT assay results, sfu/$10^6$ PBMCs[a] | | | Cultured IFN-γ ELISPOT assay results, sfu/$10^6$ cultured cells[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pp65 | gB | IE1 | pp65 | | gB | | IE1 | |
| | Week 32 | | | Week 0 | Week 32 | Week 0 | Week 32 | Week 0 | Week 32 |
| Group 1 | | | | | | | | | |
| V01OC002 | 0 | 0 | 0 | 100 | 242 | 6 | **1475** | 75 | 14 |
| V01OC005 | 8 | 10 | 12 | 0 | 42 | 50 | 83 | 42 | 25 |
| V01OC008[c] | 3 | 3 | 8 | 36 | **792** | 0 | 92 | 17 | 183 |
| V01OC009 | 3 | 1 | 8 | 0 | 6 | 50 | 6 | 250 | 192 |
| V01OC013 | 18 | 29 | 1 | 125 | **2258** | 92 | 125 | 0 | 42 |
| V01OC014 | 29 | 31 | 4 | 11 | **1967** | 50 | **4467** | 0 | 0 |
| V01OC016 | 32 | 22 | 8 | 11 | **2417** | 47 | **783** | 14 | 100 |
| V01ST001 | 12 | 23 | 17 | 6 | **358** | 217 | **608** | 75 | 89 |
| Group 2 | | | | | | | | | |
| V01ST002 | 0 | 3 | 0 | 11 | 0 | 8 | 33 | 0 | 0 |
| V01ST004 | **52** | 25 | 15 | 75 | **5017** | 56 | **775** | 14 | 58 |
| V01CO001 | 3 | 9 | 7 | 17 | 39 | 53 | 117 | 36 | 0 |
| V01FH001 | **70** | 40 | 2 | 33 | **2433** | 6 | **542** | 50 | 17 |
| V01OC021[d] | **90** | **92** | 25 | 100 | **6067** | 217 | **5575** | 111 | 150 |
| V01ST005 | 0 | 3 | 6 | 0 | 0 | 8 | 14 | 17 | 17 |
| V01FH004 | **96** | 33 | 27 | 61 | **417** | 8 | **6375** | 19 | 33 |
| V01OC022 | 27 | **53** | 0 | 3 | **4517** | 383 | **1600** | 42 | 50 |
| | Week 16[e] | | | Week 0 | Week 16 | Week 0 | Week 16 | Week 0 | Week 16 |
| Group 3 | | | | | | | | | |
| V01OC023 | 14 | ND | 0 | 33 | 58 | 333 | 538 | 8 | 11 |
| V01OC026 | **56** | ND | 0 | 28 | **1900** | 542 | **1658** | 19 | 17 |
| V01OC029 | **78** | ND | 0 | 53 | **1267** | 0 | **792** | 67 | 50 |
| V01FH005 | 5 | ND | 5 | 17 | **1542** | 58 | **2100** | 25 | 108 |
| V01OC030 | 8 | ND | 5 | 8 | 0 | 0 | 3 | 0 | 6 |
| V01ST006 | 8 | ND | 0 | 11 | **583** | 17 | **950** | 33 | 92 |

[a] A subject was considered to be a responder to the cytomegalovirus (CMV) proteins phosphoprotein 65 (pp65), glycoprotein B (gB), or immediate-early antigen 1 (IE1) by ex vivo interferon (IFN)–γ enzyme-linked immunospot (ELISPOT) assay if the mean no. of spot-forming units per $1 \times 10^6$ peripheral blood mononuclear cells (PBMCs) for replicate wells was ⩾50 and the mean no. of spot-forming units per well for replicate wells was greater than twice the mean background (without peptide) level. Positive responses are shown in boldface type. By use of the established cutoff in assay qualification studies, 63 CMV-seronegative subjects were negative by the assay (100% specificity), and 50 CMV-seropositive subjects were positive for at least 1 of the 3 antigens by the assay (100% sensitivity); positive responses for the CMV-seropositive subjects ranged from 50 sfu/$1 \times 10^6$ PBMCs to the upper limit of the assay, 6000 sfu/$1 \times 10^6$ PBMCs (authors' unpublished data).

[b] A subject was considered to be a responder to pp65, gB, or IE1 by the cultured ELISPOT assay if the no. of spot-forming units per $1 \times 10^6$ cultured cells was >250 and the no. of spot-forming units per well was >2 times that in wells without peptide. Data from the analysis of PBMCs from CMV-seronegative subjects before administration of the CMV DNA vaccine suggested that some subjects may have had low levels of cross-reactive T cell responses to peptides in the pools (data not shown); therefore, the criteria for a positive response to the vaccine included the requirement that the no. of spot-forming units per $1 \times 10^6$ cultured cells was >2 times that at baseline. Positive responses are shown in boldface type. By use of these criteria, no positive responses were detected for PBMCs stimulated with the IE1-specificity control peptides (IE1 is not encoded in the vaccine; for CMV-seronegative subjects vaccinated with VCL-CB01, $n = 22$; specificity for the vaccine response was 100%). The lack of IE1 responses in PBMCs from CMV-seronegative subjects up to week 32 of the study suggests that none of these subjects acquired CMV during the course of the trial.

[c] Week 0 PBMCs were not available; week 2 PBMCs were used in the assay.

[d] Week 32 PBMCs were not available; week 24 PBMCs were used in the assay.

[e] Results from the initial evaluation by the ex vivo ELISPOT assay in which pp65 peptides were split into 2 pools each are reported. The data are the sums of the no. of spot-forming units per $1 \times 10^6$ PBMCs for pool I and pool II.

The protracted time to peak responses was unexpected and may be related to the mechanism of action for DNA vaccines, which is likely to differ from that of conventional vaccines. In theory, in humans DNA vaccines produce low levels of immunogen for extended periods of time after vaccination [18]. In the absence of a large bolus of antigen, the immune response may not produce large effector responses but could prime antigen-specific memory cells. Indeed, DNA vaccines are used for the priming doses of several heterologous prime-boost vaccine regimens currently being evaluated in humans for the prevention of HIV infection and malaria [19, 20, 21]. With those vaccines, immune responses primed with DNA vaccine–encoding pathogen-derived proteins are boosted to higher levels with a viral-vectored vaccine encoding the same proteins. In a similar way, a CMV DNA vaccine could prime for a memory response that is boosted on exposure to CMV during a primary infection or on reactivation, resulting in rapid control of the virus and protection against CMV-associated disease.

Assays that measure only effector cell function directly ex vivo may not be adequate for detecting the priming of immune responses by DNA vaccines or for assessing the potential for mounting a memory response on exposure to the pathogen targeted by the vaccine. The results from our cultured ELISPOT assay suggest that induction of a directly measurable effector response is not required for priming of antigen-specific memory T cells. In fact, memory T cell responses were detected in 5 subjects who failed to demonstrate responses by the ex vivo ELISPOT assay at any time point. Establishment of the cultured ELISPOT assay as a correlate of DNA vaccine priming of immune memory will require evaluation of the assay in the context of a trial in which a memory response could be measured. For example, administration of the live attenuated CMV Towne strain vaccine strain) to CMV DNA vaccinated subjects, while not strictly a pathogenic challenge, could be used to demonstrate CMV antigen–specific T cell and B cell memory responses to CMV infection. The administration of Towne strain vaccine was previously used to demonstrate antibody priming by a canarypox virus vaccine encoding CMV gB; antibody responses after the administration of Towne strain vaccine occurred earlier and were of higher magnitude than those induced in subjects who were not primed with the CMV gB canarypox virus vaccine [22]. A correlation between cultured ELISPOT responses before administration of Towne strain vaccine to subjects vaccinated with a CMV DNA vaccine and a memory T cell response to the same antigens after administration of Towne strain vaccine would support the use of the cultured ELISPOT assay for evaluation of the priming of memory immune responses induced by DNA vaccines.

The CMV DNA vaccine evaluated here was more effective for inducing CMV antigen–specific T cells than gB-specific antibody. The likelihood of success for a CMV DNA vaccine focused primarily on the induction of cellular immune responses in the transplant population is supported by several investigations. First, disease severity after allogeneic transplants was decreased by infusing transplant recipients with CMV-specific T cells expanded ex vivo from the donors [13, 23, 24]. Second, a live attenuated vaccine (Towne strain) was used to ameliorate disease severity in CMV-seronegative renal transplant recipients who had received an organ from a CMV-seropositive donor [25]. In these studies, the emphasis was on the prevention of disease rather than infection. Last, strong inverse correlations between the magnitude of both $CD4^+$ and $CD8^+$ T cell responses and the development of disease in transplant recipients have been reported [12, 26].

Unlike in the transplant setting, antibody responses directed to the major surface glycoproteins, especially gB, appear to be critical in preventing maternal-fetal transmission, but antibody responses do not provide complete protection against maternal-fetal transmission of CMV [15]. Infection with other CMV genotypes or reactivation of latent CMV infection can occur with subsequent transplacental transmission to the fetus. Cell-mediated immune responses to CMV immunogens, such as pp65, are also likely to play an important role in reducing the maternal viral load, which could reduce maternal-fetal transmission or the occurrence of disease.

The potential efficacy of a plasmid-based vaccine approach to the prevention of maternal-fetal transmission was recently demonstrated in a guinea pig model of transplacental transmission of guinea pig CMV (GPCMV). Administration of a DNA vaccine encoding the GPCMV gB before conception induced gB antibody responses and decreased viral loads in live-born guinea pig pups of vaccinated dams challenged with GPCMV during pregnancy [27]. A more recent study using an alphavirus replicon vaccine encoding the GPCMV homologue of pp65 in the guinea pig model indicated that T cell responses to pp65 correlated with a reduction in viral load in the peripheral blood of the dams and a reduction in pup mortality [28]. Thus, the results in the guinea pig model suggest that DNA vaccine–induced antibody and cell-mediated immune responses could reduce CMV infection and disease.

A vaccine against CMV is currently not available; however, live attenuated CMV vaccines, canarypox-vectored vaccines, and recombinant gB protein vaccines have been or are currently in clinical trials [29]. The use of a CMV DNA vaccine in immunocompromised subjects would eliminate the safety concerns of live attenuated CMV or live recombinant viral-vectored vaccines. In addition, a CMV DNA vaccine has the advantage of delivering CMV antigens while avoiding the many CMV-encoded products involved in immune evasion [30].

In summary, the results of this phase 1 clinical trial suggest that VCL-CB01, a bivalent CMV DNA vaccine, was well tolerated and immunogenic. The vaccine induced both gB antibodies and T cell responses to both pp65 and gB at a 1- or 5-mg dose on either injection schedule and the priming of memory T cells in a

majority of CMV-seronegative subjects. The safety and immunogenicity data from this trial support further clinical investigation of VCL-CB01.

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## References

1. Gershon AA, Gold E, Nankervis GA. Cytomegalovirus. In: Evans AS, Kaslow RA, eds. Viral infections of humans. 4th ed. New York: Plenum Press, **1997**:229–51.
2. Pass RF. Cytomegalovirus. In: Knipe DM, Howley PM, eds. Fields virology. 4th ed. Philadelphia: Lippincott Williams & Wilkens, **2001**:2675–705.
3. Deayton JR, Sabin CA, Johnson MA, Emery VC, Wilson P, Griffiths PD. Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy. Lancet **2004**; 363:2116–21.
4. Stagno S, Pass RF, Cloud G, et al. Primary cytomegalovirus infection in pregnancy: incidence, transmission to fetus, and clinical outcome. JAMA **1986**; 256:1904–8.
5. Stagno S. Cytomegalovirus. In: Remington JS, Klein JO, eds. Infectious diseases of the fetus and newborn infant. Philadelphia: W. B. Saunders, **2001**:389–424.
6. Stratton KR, Durch JS, Lawrence RS. In: Vaccines for the 21st century: a tool for decision making. Washington, DC: National Academy Press, **2000**:1–10, 165–171.
7. Junghanss C, Boeckh M, Carter RA, et al. Incidence and outcome of cytomegalovirus infections following nonmyeloblative compared with myeloblative allogeneic stem cell transplantation: a matched control study. Blood **2002**; 99:1978–85.
8. Junghanss C, Storb R, Maris MB, et al. Impact of unrelated donor status on the incidence and outcome of cytomegalovirus infections after nonmyeloblative allogeneic stem cell transplantation. Br J Haematol **2003**; 123:662–70.
9. Hirsch MS. Cytomegalovirus and human herpesvirus types 6, 7, and 8. In: Kasper DL, Braunwald E, Fauci AS, Hauser SL, Longo DL, Jameson JL, eds. Harrison's principles of internal medicine. 16th ed. New York: McGraw-Hill, **2005**:1049–53.
10. Nichols WG, Corey L, Gooley T, Davis C, Boeckh M. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)–seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. J Infect Dis **2002**; 185:273–82.
11. Boeckh M, Fries B, Nichols WG. Recent advances in the prevention of CMV infection and disease after hematopoietic stem cell transplantation. Pediatr Transplant **2004**; 8(Suppl 5):19–27.
12. Gamadia LE, Remmerswaal EBM, Weel JF, Bemelman F, van Lier RAW, Ten Berge IJM. Primary immune responses to human CMV: a critical role for IFN-γ-producing CD4+ T cells in protection against CMV disease. Blood **2003**; 101:2686–92.
13. Cobbold M, Khan N, Pourgheysari B, et al. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. J Exp Med **2005**; 202:379–86.
14. Sylwester AW, Mitchell BL, Edgar JB, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med **2005**; 202:673–85.
15. Fowler KB, Stagno S, Pass RF. Maternal immunity and prevention of congenital cytomegalovirus infection. JAMA **2003**; 289:1008–11.
16. Selinsky C, Luke C, Wloch M, et al. A DNA-based vaccine for the prevention of human cytomegalovirus-associated diseases. Hum Vaccin **2005**; 1:16–23.
17. Goonetilleke N, Moore S, Dally L, et al. Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes. J Virol **2006**; 80:4717–28.
18. Doh SG, Vahlsing HL, Hartikka J, Liang X, Manthorpe M. Spatial-temporal patterns of gene expression in mouse skeletal muscle after injection of lacZ plasmid DNA. Gene Ther **1997**; 4:648–63.
19. Douek DC, Kwong PD, Nabel GJ. The rational design of an AIDS vaccine. Cell **2006**; 124:677–81.
20. Hanke T, McMichael AJ, Dorrell L. Clinical experience with plasmid DNA- and modified vaccinia virus Ankara-vectored human immunodeficiency virus type 1 clade A vaccine focusing on T-cell induction. J Gen Virol **2007**; 88:1–12.
21. Keating SM, Bejon P, Berthoud T, et al. Durable human memory T cells quantifiable by cultured enzyme-linked immunospot assays are induced by heterologous prime boost immunization and correlate with protection against malaria. J Immunol **2005**; 175:5675–80.
22. Adler SP, Plotkin SA, Gonczol E, et al. A canarypox vector expressing cytomegalovirus (CMV) glycoprotein B primes for antibody responses to a live-attenuated CMV vaccine (Towne). J Infect Dis **1999**; 180:843–6.
23. Peggs KS, Verfuerth S, Pizzey A, et al. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. Lancet **2003**; 362:1375–7.
24. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med **1995**; 333:1038–44.
25. Plotkin SA, Higgins R, Kurtz JB, et al. Multicenter trial of Towne strain attenuated virus vaccine in seronegative renal transplant recipients. Transplantation **1994**; 58:1176–8.
26. Reusser P, Cathomas G, Attenhofer R, Tamm M, Thiel G. Cytomegalovirus (CMV)-specific T-cell immunity after renal transplantation mediates protection from CMV disease by limiting the systemic virus load. J Infect Dis **1999**; 180:247–53.
27. Schleiss MR, Bourne N, Bernstein DI. Preconception vaccination with a glycoprotein B (gB) DNA vaccine protects against cytomegalovirus (CMV) transmission in the guinea pig model of congenital CMV infection. J Infect Dis **2003**; 188:1868–74.
28. Schleiss MR, Lacayo JC, Belkaid Y, et al. Preconceptual administration of an alphavirus replicon UL83 (pp65 homolog) vaccine induces humoral and cellular immunity and improves pregnancy outcome in the guinea pig model of congenital cytomegalovirus infection. J Infect Dis **2007**; 195:789–98.
29. Schleiss M. Progress in cytomegalovirus vaccine development. Herpes **2005**; 12:66–75.
30. Mocarski ES Jr. Immumodulation by cytomegalovirus: manipulative strategies beyond evasion. Trends Microbiol **2002**; 10:332–9.