HUMAN CYTOMEGALOVIRUS (HCMV) IMMUNITY IN NATURALLY SEROPOSITIVE AND VACCINATED INDIVIDUALS

Ph.D. Thesis

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Contents

Publications with results incorporated in the thesis

I. **Gyulai Zs,** Endresz V, Burian K, Pincus S, Toldy J, Cox W.I, Meric C, Plotkin S.A, Gonczol E, Berencsi K: Cytotoxic T lymphocyte (CTL) responses to human cytomegalovirus pp65, IE1-exon4, gB, pp150, and pp28 in healthy individuals: reevaluation of prevalence of IE-specific CTLs. J Infect Dis 2000; 181 (5): 1537-46 IF: 4.966

II. Adler S.P, Plotkin S.A, Gonczol E, Cadoz M, Meric C, Wang J.B, Dellamonica P, Best A.M, Zahradnik J, Pincus S, Berencsi K, Cox W.I, **Gyulai Zs**: A canarypox vector expressing cytomegalovirus (CMV) glucoprotein B primes for antibody responses to a live attenuated CMV vaccine (Towne). J Infect Dis. 1999; 180 (3): 843-846 IF: 4.966

III. Berencsi K, **Gyulai Zs**, Gonczol E, Pincus S, Cox W.I, Michelson S, Kari L, Meric C, Cadoz M, Zahradnik J, Starr S, Plotkin SA: A canarypox vector expressing cytomegalovirus phosphoprotein 65 (pp65) induces long-lasting cytotoxic T cell responses in human cytomegalovirus (HCMV) seronegative volunteers. J Infect Dis. 2001; 183 (8): (accepted) IF: 4.966

Further publications related to the thesis

IV. Berencsi K, Gonczol E, Endrész V, Kough J, Takeda S, **Gyulai Zs**, Plotkin SA, Rando RF: The N-terminal 303 amino acids of the human cytomegalovirus envelope glycoprotein B (UL55) and the exon 4 region of the major immediate early protein 1 (UL 123) induce a cytotoxic T-cell response. Vaccine 1996; 14 (5): 369-374 IF: 2.234

V. Endrész V, Kari L, Berencsi K, Kari Cs, **Gyulai Zs**, Jenei Cs, Pincus S, Rodeck U, Meric C, Plotkin SA, Gonczol E: Induction of human cytomegalovirus (HCMV)-glycoprotein B (gB)- specific neutralizing antibody and phosphoprotein 65 (pp65)-specific cytotoxic T lymphocyte responses by naked DNA immunization. Vaccine 1999; 17 (1): 50-58 IF: 2.234

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II. Berencsi K, **Gyulai Zs,** Endresz V, Hegedus K, Burian K, Cox WI, Pincus S, Plotkin SA, Gonczol E: Different phenotype of HCMV-IE, pp150, pp65 and gB-specific CTL. Abstracts of the 22nd. International Herpesvirus Workshop, La Jolla, CA. USA. p.551 (1997)

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X. Berencsi K, **Gyulai Zs**, Pincus S, Cox WI, Meric C, Cadoz M, Zahradnik J, Gonczol E, Starr S, Plotkin SA: Induction of human cytomegalovirus (CMV) specific cytotoxic T lymphocyte and helper T cell responses in humans by a canarypox-CMV-phosphoprotein 65 (pp65) recombinant. 7th. International Cytomegalovirus Workshop, Brighton, Great Britain. J Clinical Virology 1999; 12 (2): 165

XI. Burian K, Endresz V, **Gyulai Zs**, Virok D, Pincus S., Cox WI, Meric C, Plotkin SA, Berencsi K, Gonczol E: Prevalence of the IE1-exon 4-specific CTL in naturally seropositive healthy individuals. 7th. International Cytomegalovirus Workshop, Brighton, Great Britain. J Clinical Virology 1999; 12 (2): 141

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Abbreviations

ALVAC	canarypox virus
B-LCL	B-lymphoblastoid cell lines
CPE	cytopathic effect
CTL	cytotoxic T lymphocyte
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FCS	fetal calf serum
HCMV	human cytomegalovirus
HIV	human immundeficiency virus
HLA	human lymphocyte antigen
IL-2, IL-7	interleukin-2, interleukin-7
KIR	killer inhibitory receptor
LDA	limiting dilution assay
LIR	leukocyte immunoglobulin-like receptor
MCMV	murine cytomegalovirus
MHC	major hystocompatibility complex
MOI	multiplicity of infection
NA	neutralizing antibody
NK cell	natural killer cell
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PFU	plaque forming unit
SI	stimulation index
TAP	transporter associated with antigen processing
$TCID_{50}$	50% tissue culture infectious dose

1. Summary

Both cellular and humoral immunity are important defense mechanisms against human cytomegalovirus (HCMV) diseases. The major component of the humoral immune response consists of neutralizing antibodies to envelope glycoproteins, primarily glycoprotein B (gB). Of the cell mediated immune responses the major histocompatibility complex (MHC) class I restricted cytotoxic T lymphocyte (CTL) response to HCMV assists in the prevention of progress of virus infection to a severe or life threatening disease. Thus, an effective HCMV vaccine will need to induce not only humoral, but also a specific CTL response.

In our study the prevalence of HCMV-pp65, pp150, IE1-exon4, gB and pp28-specific CTL responses of 34 healthy, human lymphocyte antigen (HLA) haplotyped, racially and geographically diverse individuals was compared. Regardless of race or geographical location, the moderately and highly seropositive donors showed predominantly pp65- and IE1-exon4-specific CTL responses (92% and 76% of the donors, respectively), indicating the high prevalence of memory CTL to these two proteins. In addition, highly seropositive and a few moderately seropositive donors showed CTL responses to gB and pp150 (33% and 30% of the donors, respectively). No individual recognized pp28 as a CTL target. The precursor frequency of IE1-exon4 and pp65-specific CTL was analyzed by limiting dilution assay (LDA) and found comparable in the donors tested. Antigen presenting alleles were determined for pp65 as A1, A2, A28, A68, B7, B12/44, for IE1-exon4 as A1, A2, A3, B7, B8, B18, B70, and for pp150 as A3, B14. Analysis of the restricting allele for gB-specific effector cells identified the class II antigens DR7, DR15, and DQ2 or 6. Phenotypic analysis of pp65-specific effector cells revealed mixed effector population of CD4+ and CD8+ cells in one donor, while analysis of pp65-specific effector cells from another two donors showed that effector cells were CD8+ cells. The CTL response was exerted mainly through CD8+ effector cells specific for pp150 and IE1-exon4, whereas, gB-specific CTL were CD4+.

To develop a vaccine against HCMV, two canarypox-HCMV recombinants were constructed and examined by us for their ability to induce HCMV-specific immune responses in randomized, double-blind, phase I clinical trials.

Further, a canarypox virus expressing HCMV gB was evaluated in combination with a live, attenuated HCMV vaccine (Towne). To determine whether canarypox-CMV-gB could

prime for antibody responses, 20 seronegative adults randomly received either $10^{6.8}$ $TCID_{50}$ (50% tissue culture infectious dose) of canarypox-CMV-gB or $10^{6.8}$ $TCID_{50}$ of canarypox-RG, expressing the rabies glycoprotein, administered at 0 and 1 month, with all subjects receiving a dose of $10^{3.5}$ pfu (plaque forming unit) of the Towne vaccine at 90 days. Subjects primed with canarypox-CMV-gB, developed neutralizing titers and ELISA antibodies to HCMV-gB sooner, the antibody titers were much higher, and persisted longer than for subjects primed with canarypox-RG.

As shown by us, and other investigators, the major matrix phosphoprotein (pp65) is an important target of HLA-restricted CTL after natural infection, thus a canarypox-CMV-pp65 recombinant was examined for its ability to induce HCMV-pp65 specific CTL, helper T lymphocytes, and antibodies in immunized volunteers. Twenty-one HCMV seronegative volunteers were randomized to receive bilateral intramuscular immunizations at months 0, 1, 3 and 6 with either $10^{6.8}$ $TCID_{50}$ of canarypox-CMV-pp65, or placebo. In canarypox-CMV-pp65 immunized individuals pp65-specific CTL were elicited after only 2 vaccine doses and were present at month 12 and 26 in all of the tested volunteers. Cell-depletion studies from 11 pp65 CTL responders indicated that the CTL were of the CD8+ phenotype. Peripheral blood mononuclear cells (PBMC) of the immunized volunteers proliferated in response to stimulation with purified pp65, and antibodies specific for pp65 were also detected in the vaccinees.

Canarypox-CMV-pp65 is the first recombinant vaccine to elicit HCMV-specific CTL responses in humans. These results, taken together with the observation that a canarypox-CMV-gB recombinant has a priming effect on neutralizing antibody responses in humans, lead us to conclude that canarypox-HCMV recombinants can stimulate both arms of the immune response, and are promising candidates for immunization against HCMV diseases.

2. *Introduction*

2.1. ***Human cytomegalovirus (HCMV)*** is a member of the herpesviridae family and is species-specific, so that replication occurs only in human cells [125, 126]. HCMV is a large virus, containing 230 to 240 kb of DNA in its genome, which encodes at least 190 genes [70]. An icosahedral matrix, and a lipid envelope containing glycoproteins surround the double-stranded DNA genome. Primary infection is transmitted by salivary secretions [47], by breast milk [111], by cervical secretions at birth [101], by sexual intercourse [22] and by blood transfusion [132]. Infections with HCMV are widespread (infects 50-100% of the human population), but the outcome of the infection depends greatly on the immune status of the infected person. Under normal conditions primary infections are usually asymptomatic or result in a self-limited infectious-mononucleosis-like syndrome, however, the incidence and spectrum of disease in newborns and immunocompromised hosts establish this virus an important human pathogen. Following primary infection, HCMV establishes a lifelong latency in circulating monocytes [117], bone marrow progenitor populations [54, 41], including circulating CD34+ [68] and CD14+ cells [12], and probably endothelial cells [43]. HCMV has also been suggested to be an important cofactor in the development of atherosclerosis [67, 9] and restenosis after angioplastic surgery [109, 133].

The infectious agent most frequently causing congenital malformations is the HCMV. HCMV infection is found in 0.5 to 2.5% of newborns, and about 10 to 15% of the infected children suffer either from immediate symptoms or late sequelae of the infection. Clinically apparent infections after birth are characterized by cytomegalic inclusion diseases in about 5% of the infected children. Another 5% have atypical involvement, usually with some damage of the central nervous system and 90% have no symptoms at birth. Among the most severely affected children mortality may be 30%. About 10 to 15% of the children who are infected but asymptomatic at birth eventually develop mental retardation, chorioretinitis, microcephaly, and hearing loss [16, 30].

Similarly, HCMV related complications of bone marrow and organ transplantation are quite common, the incidence of diseases attributable to HCMV ranges between 20-60%. The illness commonly takes the form of interstitial pneumonia, but hepatitis, nephritis, encephalitis, bone marrow depression, and potentiation of bacterial and fungal infections pose

additional serious problems [36, 44]. In addition, HCMV infections will develop in approximately 20% of individuals seropositive for the human immunodeficiency virus (HIV) after their CD4 levels drop to below 100/μl [26].

The congenitally infected children who are symptomatic at birth or develop severe symptoms later are usually born to mothers who sustained primary HCMV infection during pregnancy. Although some infants born to seropositive mothers may also excrete HCMV at birth, that class of infection, which may be the result of reactivation in the mother, is less likely to cause damage [110, 30]. Likewise, the most important contributing factor to the development of disease in the posttransplant period is the serologic status of the donor and recipient, the organ obtained from a seropositive donor leads to the development of serious, sometimes life-threatening diseases in seronegative, and mild symptoms in seropositive recipients [36]. These observations indicate that HCMV-immunity is protective against HCMV-caused diseases. However the virus has evolved various molecular mechanisms to evade immune surveillance, is not eliminated from the body after primary infection but persists in the form of a low-grade chronic infection or remains in a latent state, such that reactivation or increased excretion of virus later allows further transmission of the virus to new hosts.

The relative significance of cellular and humoral immunity in the defense mechanisms against HCMV disease is uncertain. However, after bone marrow, peripheral blood stem cell or renal transplantation, the recovery of CD8+ class I MHC-restricted CTL response to HCMV correlates with an improved outcome from HCMV disease [91, 89, 97-99, 59]. For example, HCMV interstitial pneumonia has been observed only in patients with undetectable levels of CD8+ HCMV-specific CTL [99]. Specific adaptive immune therapy with CD8+ T cell clones has also provided support for the importance of CTL in protection against HCMV disease: none of 5 bone marrow recipients who received T cell infusions to reconstitute HCMV immunity developed HCMV viremia or HCMV disease after initiating T cell therapy [103, 123]. Further, it appears that infants chronically excreting HCMV after congenital infection at birth cease to excrete the virus when HCMV-specific cell mediated immunity appears later in life [112]. These results suggest that T cell responses are important in HCMV immunity and that strategies developed by HCMV to escape immune surveillance may rely in the reduction of cell mediated immunity, especially cytotoxic T cell function. Indeed, the

HCMV genome contains at least 4 genes (US3, US6, US2, US11) involved in down-regulating the surface expression of HLA class I molecules, HCMV also sequesters CC chemokines, induces Fc receptors, interferes with induction of HLA class II antigens, and inhibits natural killer (NK) cell activity [69]. HCMV pp65 can also block the presentation of immediate early (IE) antigens, the first viral proteins to be produced [35]. The US3-gene product gpUS3 is an endoplasmic reticulum (ER) resident protein which retains stable, peptide-loaded MHC class I molecules in the ER compartment [49]. gpUS6, another early ER resident, transmembrane protein coprecipitates as a TAP (transporter associated with antigen presentation)-β_2-microglobulin-class I complex. gpUS6 associated with TAP prevents peptide translocation [3]. gpUS2 and gpUS11 are involved in the downregulation of HLA class I molecules as well, they cause N-glycanase- and SEC61-mediated retrogradation of HLA-I from ER to the cytosol where they are degraded by proteasomes [129, 62]. All of these mechanisms result in a decreased HLA class I cell surface expression, which can be sensed by killer inhibitory receptors (KIR) on NK cells. However, HCMV also evolved strategies to counteract NK susceptibility. HCMV also encodes a homologue of the MHC I heavy chain (gpUL18), which binds to β2-microglobulin and inhibits NK cell activity through selective binding to a leukocyte immunoglobulin-like receptor (LIR)-type inhibitory receptor [100]. Another potential target for HCMV to control NK cell mediated lysis is the inhibitory receptor CD94/NKG2A that shows a broad distribution on NK cells in peripheral blood. The only ligand known, so far, for the CD94/NKG2A heterodimer is HLA-E, which is expressed on virtually all cells. The UL40 gene of HCMV encodes a canonical ligand for HLA-E, thus expression of UL40 in HLA-E –positive target cells confers resistance to NK cell lysis [118]. The decreased surface levels of MHC class I molecules reduce T cell recognition of HCMV proteins, and might explain the incomplete immunity, probably incomplete CTL responses and consequently, the virus-persistence after natural infection.

Thus, the ideal HCMV vaccine should be more effective than natural immunity, or immunization with live attenuated HCMV strains, and should prevent infection as well as disease in order to prevent establishment of viral persistence. Such a vaccine would prevent virus attachment/penetration and spread by inducing the production of neutralizing antibodies through the activation of B and T helper cells. It would also prevent virus replication in

infected cells by activating cytotoxic T lymphocytes specific for viral proteins present in the cells early in infection.

CTLs appear to be the major T-cell effectors of antiviral activity [23, 50, 71, 73, 116]. The CTL receptor recognizes a short peptide derived from an endogenously produced viral protein in the context of the MHC class I β2-microglobulin heterodimer expressed on the surface of an infected cell. Because the MHC class I restriction elements are present in relatively high concentration on almost all nucleated cells except neurons, CTLs can exert their antiviral effects against most infected cells. In most instances, antigen presentation by the MHC class I β2-microglobulin heterodimer is restricted to viral peptides that are produced and processed during infection. Hence, CTLs cannot function to prevent infection, but instead, are limited to the elimination of cells already infected or, alternatively, to the restriction of virus replication by the elaboration of antiviral cytokines. The net effect of CTL activity is to prevent further spread of virus and to terminate infection in cells already infected. Because of the important role of CTLs in clearance of established viral infections, the incorporation of CTL epitopes into viral vaccines has been considered an important requirement.

Studies to identify which HCMV antigens might be important in immunity have demonstrated that the lower matrix protein pp65 is the immunodominant target for HCMV-specific CTL [103, 34, 66, 130, 13]. Indeed, high pp65-specific CTL precursor frequencies were demonstrated in seropositive individuals [130, 13]. Epitope specificity of pp65-specific CTL and the presenting HLA alleles were also identified in some individuals [66, 130, 24]. HCMV IE1-, pp150-, gB-, and/or gH-specific CTL have also been detected in studies on a limited number of individuals, but the data are scarce and the prevalence of specific CTL remains controversial [103, 130, 13, 14, 5, 120, 35, 46, 51]. To our knowledge, the only study to examine CTL responses specific to pp28, which is a potent inducer of antibody production [55], reported the absence of these CTL in all 4 seropositive individuals tested [13]. At least some of the conflicting results might rest in the haplotype differences that effect antigen presentation in different racial groups, and the dominance of CTL epitope variations in HCMV strains in one geographical area but not in another.

Fewer data are available about the role of CD4+ T helper cells in HCMV infection. Several HCMV proteins (pp65, p52, gB, gH, IE1, IE2, pp71) have been identified as target antigens for lymphocyte proliferative i.e. T_H cell responses [5, 42, 7, 121, 29, 52]. In

comparative analyses of fourteen and four HCMV proteins pp65 represented the dominant antigen recognized by T helper cells [7, 121].

2.2. *Vaccination against cytomegalovirus*

Different approaches were taken to develop a vaccine against HCMV infection.

Attenuated, live vaccines

Two live virus vaccines were developed, one in the United Kingdom and one in the United States. Elek and Stern used the AD-169 laboratory strain to immunize normal adults [25, 72], but vaccination strategies with this strain have not been further investigated. Plotkin and colleagues isolated the Towne strain from the urine of a congenitally infected child, whose name was Towne, and passaged the new strain in human embryo fibroblasts until the 125^{th} passage [83].

Towne is a classic live attenuated virus that when used as a vaccine strain in a phase-1 clinical trial induced seroconversion including neutralizing antibody, lymphocyte proliferative responses and HLA restricted cytotoxicity - although, unlike after natural infection it tended to fade by 1 year postvaccination. Vaccinees developed only slight and tolerable local and systemic symptoms. Attempts to recover virus from those recently vaccinated were uniformly negative [65, 90, 1].

As shown in three randomized, controlled, double blind studies in seronegative renal allograft recipients, protection against natural challenge was incomplete after immunization with the Towne vaccine. Although vaccination did not prevent infection, it did decrease frequency and mitigate severity of HCMV induced disease. The vaccine virus failed to reactivate in the immunosuppressed host, suggesting that it had not become latent [84-87].

Healthy seronegative individuals immunized with the HCMV Towne strain and challenged with the low-passage Toledo strain (an isolate from the urine of a congenitally infected infant from Toledo) in graded doses were resistant to disease but not against challenge infection in a dose-dependent manner, indicating incomplete immunity if the challenge dose was 100 pfu. Vaccine-induced immunity to HCMV was as complete as naturally induced immunity when the challenge dose of Toledo was 10 pfu [88].

Vaccination with the Towne strain failed to prevent natural child-to-mother transmission of HCMV, while women who were naturally seropositive were protected [2]. However, due to the low number of participants in this trial, congenital infection or congenital malformation induced by the natural challenge virus could not be evaluated.

Improved versions of theTowne strain

Researchers at Aviron pharmaceutical company noticed that a Towne vaccine virus variant had a deletion of 13 kb of DNA when compared with the Toledo wild virus. Sequencing of the deletion revealed 19 open reading frames presumably coding for proteins. Although there are other differences between the two genomes, they hypothesized that the 19 open reading frames missing from Towne contribute to virulence and immunogenicity and that restoration of some or all of those genes might result in a more immunogenic vaccine [18]. Hybrids between Towne and Toledo have been constructed, and these hybrid viruses are presently under investigation in humans to determine if induction of immunity has been enhanced. If successful, this approach would yield a mutant strain that might duplicate natural infection but still is attenuated.

Subunit glycoprotein B

The epitopes that induce neutralizing antibodies are present on the envelope glycoproteins of HCMV, of which there are three major groups (gB, gH and gcII). The best studied is the gB protein, which is responsible for at least one-half of neutralizing antibodies in the serum of naturally infected individuals [38].

The gene for gB was inserted into Chinese hamster ovary cells, obtaining a stably transfected cell line, and the 3' end of the gene was truncated to obtain better secretion of gB [107]. Because in preclinical immunization experiments the antibody levels induced by the vaccine formulated with the standard aluminum hydroxide gel as adjuvant were low, an oil-in-water adjuvant called MF-59 was used for immunization of humans. The results of immunization with HCMV-gB showed a high neutralizing antibody response after three doses of antigen with MF-59 adjuvant, that exceeded those in seropositive control subjects [79, 31]. The antibodies elicited by gB neutralized all primary HCMV isolates tested [60], however, it was noted that the levels of neutralizing antibodies fell rapidly during the 6 months after the

third dose, therefore a fourth dose was administered, resulting in an anamnestic response that seemed to have greater persistence.

Table 1. Cytomegalovirus proteins that might be included in a vaccine

		Human immune response	
Proteins	**Molecular size**	**Neutralizing antibody**	**Cytotoxic T cells**
Envelope glycoproteins			
gB	55-130 kDa	+	+
gH	85-145 kDa	+	+
gcII	47-52 kDa	+	?
Structural proteins			
Lower matrix	65-71 kDa	-	+
Major nucleocapsid	150 kDa	-	+
28-32 kDa	28-32 kDa	-	-
Nonvirion			
IEI	72 kDa	-	+

DNA plasmids

A new strategy for immunization involves direct inoculation of plasmid DNA encoding viral protective antigens into tissues *in vivo* [131, 119]. Expression of the antigen is driven by a strong promoter capable of efficient activity in a variety of mammalian cell types. Although there are several safety issues yet to be resolved, DNA plasmids are attractive immunogens and are particularly adept in inducing cellular immunity. Therefore the first studies of plasmids containing HCMV genes have been undertaken in mice with pp65 and gB as the encoded antigen [Attachment V, 75]. Our group reported that the gB and pp65 proteins, as expressed by mammalian expression plasmids inoculated alone or in combination, induce gB and pp65-specific antibody and pp65-specific CTL responses in mice.

Recombinant viruses

Viral vectors can be engineered to carry heterologous peptides or proteins from other microorganisms. Work in our laboratory aimed at developing a recombinant vaccine to HCMV has shown that the gB protein when expressed by adenovirus [64], vaccinia virus [38], or canarypox (ALVAC) virus [37] vectors induces neutralizing antibodies in experimental animals, lymphocyte proliferation upon *in vitro* antigen stimulation [10], and CTL responses in mice of H-2k haplotype [11]. We have also shown that mice immunized with adenovirus recombinant viruses expressing truncated forms of the gB gene containing the first 700, 465, or 303 amino acids of gB or an adenovirus construct containing exon4 of the HCMV immediate early 1 gene demonstrate HCMV-specific CTL responses [Attachment **IV**].

To date, most success has been achieved using poxviruses as recombinant vectors. The poxviruses, of which vaccinia is a prototype, are known for their ability to induce strong cellular immune responses [8]. Foreign DNA sequences can be inserted into the very large genome of poxviruses and the proteins coded by the sequences are expressed [78, 63]. However, vaccinia virus can replicate unchecked in immunocompromised hosts and is associated with adverse reactions [56]. Avian poxviruses are safe alternatives to vaccinia, and canarypox virus was identified as a useful vector, because it can be grown in avian cells but does not replicate in mammalian cells, providing a large safety factor [114]. Despite its inability to produce infectious virions in mammals, canarypox does transcribe and translate information from early genes and if DNA sequences encoding antigenic proteins of vaccine interest are inserted downstream of early promoters, their genetic information will also be expressed. Furthermore, live canarypox has been administered to immunosuppressed mice and HIV-infected humans without serious adverse effects [113, 82].

Recombinant viruses based on an attenuated strain of canarypox virus (ALVAC) have been constructed [6, 113, 82] and have been shown to be well tolerated in humans and to induce antibody responses against many viral antigens, including rabies virus glycoprotein [17, 33], measles virus hemagglutinin and fusion proteins [115], HIV-1 envelope glycoproteins [81], and CTL responses specific to HIV glycoproteins in about 30%-39 % of the vaccinated individuals [81, 21]. In addition, immunization with an ALVAC vector expressing multiple HIV-1 genes followed by a boost with recombinant gp120 resulted in durable CTL responses to all of the encoded genes [27]. Concerning HCMV, although

immunization with ALVAC-CMV-gB recombinants has resulted in induction of neutralizing antibody to HCMV in laboratory animals, serologic responses to date in human volunteers have been weak. A similar situation has been observed for HIV. Two doses of an ALVAC expressing HIV glycoproteins induced poor serologic responses in human volunteers, but when boosted with purified HIV glycoproteins a markedly enhanced antibody response was observed [21].

2.3. *Aims*

The present study was designed to address the following aims:

Aim 1. To determine the prevalence and characteristics of pp65-, pp150-, IE1-, gB- and pp28-specific CTL responses in a randomly selected racially and geographically divergent population of healthy, naturally HCMV seropositive individuals.

Aim 2. To determine the tolerability and the immunogenicity of the ALVAC-CMV-gB recombinant in combination with the Towne vaccine in humans.

Aim 3. To determine whether an ALVAC-CMV-pp65 recombinant vaccine is well tolerated in humans and whether this recombinant induces long lasting pp65-specific CTL, helper T cell responses and antibodies in a heterologous HCMV seronegative population comparable to that of naturally seropositive individuals.

3. *Materials and Methods*

3.1. *Study population*

Thirty-four healthy adults enrolled in the blood donor program of the Wistar Institute (Philadelphia, PA., USA) and the Albert Szent-Györgyi Medical University (Szeged, Hungary) were studied. The group was composed of 12 blacks, 1 Chinese, 1 Japanese, and 20 whites. Age range of the subjects was 22-60 years.

3.2. *HLA typing*

Peripheral blood mononuclear cells (PBMC) were typed for major histocompatibility complex (MHC) class I and class II antigens by standard complement-mediated cytotoxicity assay and by polymerase chain reaction (PCR), which were performed by the Immunology and Histocompatibility Laboratory of the Hospital of the University of Pennsylvania, Philadelphia, and by the South Hungarian Regional Blood Bank of National Transfusion Service, Szeged. [**I**/ Table 1] lists the characteristics of each donor.

HCMV seropositivity was determined by microneutralization assay [39] and by standard ELISA.

3.3. *Microneutralization assay*

Serial 2-fold serum dilutions were prepared in culture medium (MEM+7.5% fetal calf serum) in duplicate wells of sterile, 96 well, flat-bottomed microtest culture plates. 2000 pfu. of HCMV and 0.005 ml of guinea pig complement (Bio-Whittaker Walkersville, MD) were added to each well. After 1-h incubation period at 37°C in a CO_2 incubator, MRC5 cell suspension (3-5 x 10^4 cell/well) was added and the plates were further incubated for 2-3 days. The results were read as reciprocals of the highest dilution of serum that fully inhibited viral cytopathic effect (CPE) compared to 100% CPE in virus control wells. A reference human serum with known neutralizing titer and a neutralization-negative human serum were included in each assay as positive and negative controls. Using a standard amount of a titrated, aliquoted virus pool, the positive control serum gave the same neutralization titer in each assay and the negative serum did not react. Sera with a neutralizing antibody (NA) titer of <12 were considered negative, titers 1:12-1:96 were moderately seropositive and titers ≥1:128 were highly seropositive.

3.4. *ELISA assay*

Purified HCMV was used as coating antigen, and lysate of uninfected MRC5 cells served as control antigen. For each serum dilution, optical density values obtained in control antigen coated wells were subtracted from those obtained in HCMV antigen coated wells and were considered positive at OD values $\geq$0.2 higher than the reference negative serum at a dilution of 1:100.

For pp65-specific ELISA assay lysate of 293 cells transiently transfected with pΔRC-pp65 was used as coating antigen, lysate of untransfected 293 cells served as control antigen [Attachment **V**]. pp65-specific OD values were calculated for each serum dilution: optical density values obtained in control antigen-coated wells were subtracted from those obtained in pp65 antigen-coated wells. Sera with OD values > 0.053 (mean OD of serum 200x dilution+2x SD of originally seronegative subjects at M. 0) were considered positive for pp65 antibody.

For gB-specific ELISA, plates were coated with 150 ng/well purified gB (Chiron Corporation Emeryville, CA). Sera with OD values >1.00 at serum dilution of 1:200 were considered positive for gB antibody.

3.5. *Viruses*

Human HCMV strain Towne was propagated and titered on MRC-5 cells (obtained from Coriell, Camden, NJ). HCMV stocks were stored at -70°C.

Canarypox (ALVAC) and vaccinia recombinant viruses were developed by collaborating partners Steve Pincus and William I. Cox at Virogenetics Corporation (Troy, NY, USA). ALVAC vaccines were manufactured by Pasteur Mérieux Connaught France (Marcy l'Etoile, France).

The pp65 gene was PCR-amplified using genomic HCMV DNA as template (Towne strain) and cloned as described [Attachment **V**]. The IE1-exon4 gene (AD169 strain) was amplified by PCR from the pJD083 plasmid [4]. The pp150 and pp28 gene was amplified from HCMV genomic DNA (Towne strain) by PCR.

The ALVAC-gB recombinant has been described [37]. ALVAC-pp65, pp150, IE1-exon4, and pp28 recombinants were constructed by placing these genes under the control of a

vaccinia virus promoter in a canarypox donor plasmid from which a nonessential gene was specifically deleted as described [37].

WR-gB, WR-pp65, IE1-exon4, and pp28 recombinants (Vac-gB, Vac-pp65, Vac-IE1, and Vac-pp28) were derived using a host range selection system [77, 80] that consists of WR virus deleted of the K1L host range gene and an insertion plasmid that contains the K1L gene and the relevant HCMV gene. Expression of inserts in all recombinants was confirmed by immunoprecipitation of radiolabeled infected cell extracts using specific monoclonal antibodies and polyacrylamide gel electrophoresis analysis. The Vac-pp150 construct was kindly provided by Stanley Riddell (Fred Hutchinson Cancer Research Center, Seattle, WA). Vaccinia viruses were propagated and titered on Vero cells (American Type Culture Collection, Rockville, MD). Canarypox viruses were grown and quantitated on primary chick embryo fibroblast cultures. Virus stocks were stored at -70°C.

Immunizations were performed with the following viruses:

In trial A, the Towne vaccine (lot C-107) was prepared by Program Resources (Rockville, MD). The mean infectivity titer was $10^{3.5}$ plaque-forming units (pfu)/lyophilized vial, which constituted a single dose. ALVAC-RG expresses the glycoprotein G gene of the ERA strain of rabies virus. Batches S3106 (ALVAC-RG), and S3145 (ALVAC-CMV-gB) with titers of $10^{6.8}$ $TCID_{50}$ per dose were used in the trial.

In trial B, immunizations were performed with batch S3227 of ALVAC-CMV-pp65. It had a titer of $10^{6.8}$ $TCID_{50}$ per dose.

3.6. *CTL assays*

3.6.1. *Target cell lines*

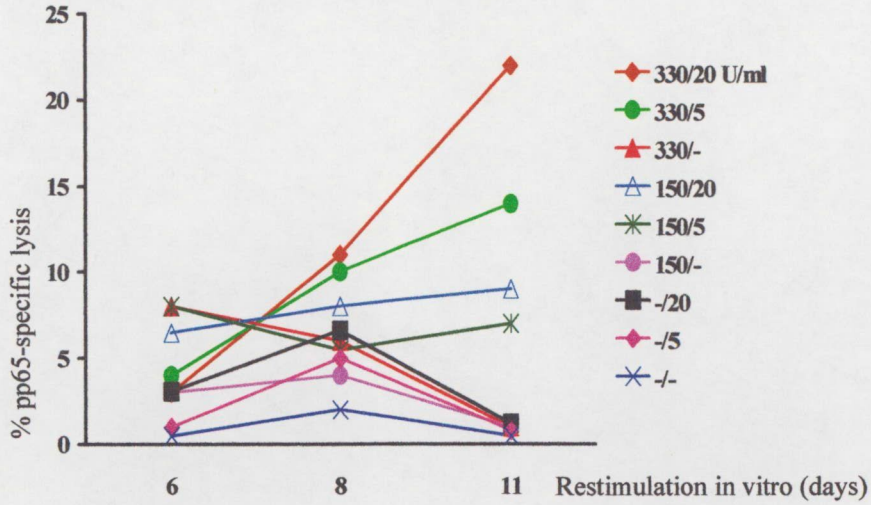
Autologous B-lymphoblastoid cell lines (B-LCL) were established from each donor by incubation of peripheral blood mononuclear cells (PBMC) with supernatant from the Epstein-Barr virus (EBV)-producing marmoset cell line B95.8 (American Type Culture Collection). Transformed cell lines were grown in RPMI 1640 (Mediatech Cellgro, Herndon, VA) with 15% fetal calf serum (FCS) (Hyclone, Logan, UT, or Cansera Atlanta Biologicals, Norcross, GA) and cryopreserved until use.

3.6.2. *Effector cells*

PBMC were separated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ.) density gradients. Freshly separated or cryopreserved PBMC were stimulated *in vitro* with autologous PBMC expressing HCMV proteins. Stimulator cells were prepared by infecting PBMC with canarypox virus vectors encoding HCMV pp65, IE1-exon4, gB, pp150 or pp28 at a multiplicity of infection (MOI) of 5. After a 1h incubation, stimulator cells, infected separately with the canarypox virus constructs or with control parental canarypox virus were washed and mixed with responder PBMC at a responder-to-stimulator ratio of 5:1. The mixture of responder and stimulator cells was placed in 24-well plates at $3.5x10^6$ cells/2ml medium /well. The culture medium was RPMI 1640 (Mediatech Cellgro, Herndon, VA), supplemented with 10% FCS (Hyclone, Logan, UT, or Cansera Atlanta Biologicals, Norcross, GA), 4 mM L-glutamine (Sigma, St. Louis, MO), 60 μg/ml gentamicin (Sigma), and 5 μM 2-mercaptoethanol (Sigma).

In our preliminary experiments PBMC were restimulated *in vitro* with the canarypox recombinants for 6 days, but only strongly seropositive individuals responded with HCMV-specific CTL. Therefore an improved *in vitro* restimulation process was developed by supplementing PBMC cultures with interleukin (IL)-7 and IL-2, and increasing the length of restimulation with canarypox recombinants. Figure 1 A shows pp65 CTL activity of donor H-34 (moderately seropositive) in the presence of different concentrations of IL-7 and IL-2. Effector cells mount significant pp65-specific CTL activity after 11 days *in vitro* culture only in the presence of 330U/ml IL-7 and 20U/ml IL-2. The influence of different concentrations of IL-7 and IL-2 on the CTL activity of a strong seropositive, strong pp65 CTL responder donor (H- 35) was also tested. Figure 1 B shows that the presence of 150-300U/ml IL-7 and 5-20U/ml IL2 was beneficial for pp65-specific CTL. In the following experiments PBMC cultures of all donors were supplemented with 330 U/ml IL-7 (R&D Systems, Minneapolis, MN) at the start of culture, and with 20 U/ml IL-2 (Genzyme, Cambridge, MA) on days 4 and 8. Cell lines were tested for CTL activity between days 8 and 13 of culture.

A.



B.

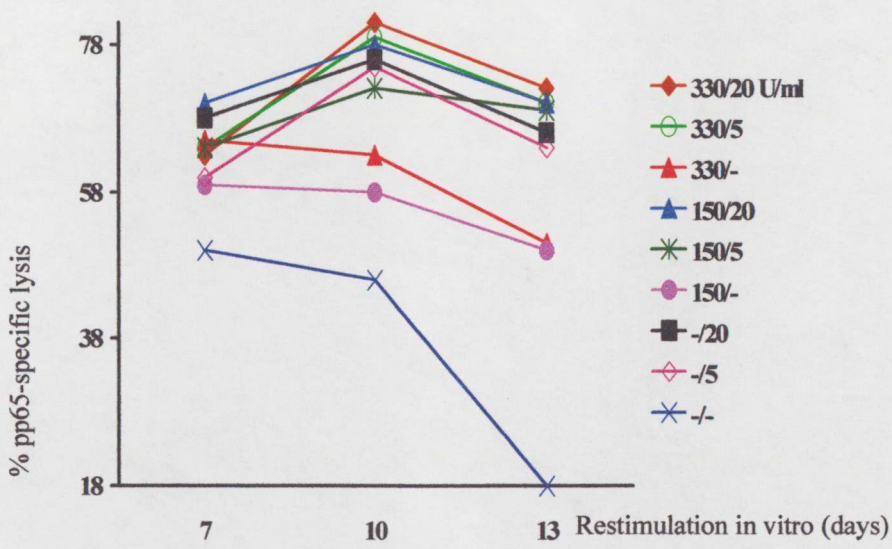


Fig. 1. Both IL-7 and IL-2 is needed for successful restimulation of pp65-specific effector cells of a weak CTL responder. PBMC of donor H-34 (moderately seropositive, weak pp65-specific CTL responder) [**A**] and of donor H-35 (strong HCMV seropositive and strong pp65 CTL responder) [**B**] were restimulated *in vitro* with ALVAC-pp65 in the presence of different concentrations of IL-7. On day 3 and 7 (in the case of donor H-34) and on day 4 and 8 (in the case of donor H-35) cultures were supplemented with different concentrations of IL-2. Effector cells were collected at times indicated and tested for pp65-specific lysis of target cells. Figure shows pp65-specific lysis of MHC-matched target cells after subtraction of background lysis.

3.6.3. *Restimulation of PBMC with HCMV-infected autologous monocytes*

Monocytes were separated from PBMC by plastic adherence: 30 x 10^6 PBMC were incubated on 96-well plates for 1 h at 37°C, nonadherent cells were aspirated, and plates were washed 3 times with warm medium. Adherent cells were infected with HCMV (MOI, 2-4 pfu/ cell). After 3-h virus adsorption, cells were washed with medium, and PBMC were added as responder cells (responder-to-stimulator ratio, 4:1). pp65- and IE1-exon4-specific lysis was tested after 8 days of *in vitro* restimulation by the use of vaccinia recombinant-infected target cells.

3.6.4. *Infection and labeling of target cells*

Each of 5 individual HCMV protein-expressing vaccinia recombinants was used to infect 2-6x10^6 target cells at MOI of 10 for 1h. Cells were diluted and incubated for an additional 16 h, washed, and labeled with 100 μCi of [^{51}Cr]$NaCrO_4$ (Amersham, Arlington Heights, IL) for 1h. Labeled cells were washed 3 times before assay.

3.6.5. *Cytotoxicity assays*

Cytolytic activity of antigen-driven effectors present in bulk cultures established from PBMC was measured in a 4-h ^{51}Cr-release assay. Nonadherent effector cells were collected, washed and plated in triplicate to round bottomed 96-well microtiter plates at effector-to-target ratios (E:T) indicated for individual experiments. Targets were autologous B-LCL or heterologous B-LCL or B-LCL matched with effectors in a single HLA class I allele. Chromium release was measured in a gamma counter (Cobra II Packard Instrument, Meriden, CT). The percentage of specific lysis was determined as follows: 100x(experimental release - spontaneous release)/(maximal release - spontaneous release). Spontaneous release was always less than 30%. Effector cells were considered positive for CTL activity if the percentage specific lysis of test wells was ≥10% above background lysis (cytotoxicity directed at WT-Vac-infected autologous target cells) at two consecutive E:T ratios. Nonspecific background cytolysis was reduced by the addition of a 30-fold excess of unlabeled, WT-Vac-infected target cells to each well of the ^{51}Cr-release assay.

3.7. *Limiting Dilution Assay (LDA)*

Effector PBMC were plated at different cell numbers in 30 replicate wells and cultured with γ- irradiated (3000 rad delivered by an MKA model 68A irradiator [Shepherd, Glendale, CA]) autologous PBMC as feeder cells ($6x10^4$ PBMC/well) and with $4x10^3$ autologous EBV-transformed lymphoblasts infected with Vac-pp65 or Vac-IE1 for 6-16h, γ-irradiated (3000 rad) and UV-irradiated, as stimulator cells. After 12-14 days, specific CTL activity of each well was determined on autologous Vac-pp65 or Vac-IE1-infected, autologous WT-Vac-infected and HLA-mismatched Vac-pp65 or Vac-IE1-infected target cells. CTL precursor frequency was estimated as the input cell number that resulted in 37% negative cultures against the pp65- or IE1-specific target [58]. Linear regression analysis was performed and precursor frequency was determined by solving the equation of the best-fit line where the fraction of nonresponding wells equaled 0.37. ^{51}Cr-release in each well was considered to be significant at >3 SD above mean lysis observed in control wells (containing only feeder and stimulator cells).

3.8. *Determination of CTL phenotype*

$3x10^6$ cell aliquots of restimulated effector cells were depleted of CD4+ lymphocytes by anti-CD4 monoclonal antibody + complement (C'), or of CD8+ lymphocytes by anti-CD8 monoclonal antibody + C', or of both CD4+ and CD8+ by both antibodies + C'. Antibodies were from Caltag Laboratories (Burlingame, CA), and were used at a predetermined concentration of 16μg/ml. Nontoxic rabbit complement (Accurate Westbury, NY) was used at a final dilution of 1:10. Effector cells were incubated with the antibodies at 4°C for 45 min., washed, and further incubated in the presence of complement for 30 min. at 37°C. Following washing, cells were resuspended in the predepletion volume of medium so nondepleted cells would remain at the original concentration, and HCMV antigen-specific lysis was determined in a 4-h ^{51}Cr-release assay.

3.9. *Lymphocyte proliferation assay*

PBMC were resuspended to a concentration of 2×10^6 cells/ml in RPMI medium supplemented with 10% autologous plasma (when available), or human AB serum (Sigma). A 100-μl aliquot containing 2×10^5 cells was added to wells of a 96-well microtiter U-bottom

plate. pp65 protein purified by high-pressure liquid chromatography from HCMV (Towne)-infected MRC-5 cells [76] or MRC-5 antigen (control antigen) at final concentrations of 2.5 μg/ml, 0.6 μg/ml, and 0.15 μg/ml in 100 μl was added to wells in triplicate. Plates were incubated at 37°C in 5% CO_2 for 6 days. In some experiments, two identical plates were set up and incubated for 5 and 6 days. [^{3}H]Thymidine (0.5 μCi) was added to each well for the last 6h of incubation. Cells were harvested with an automatic cell harvester (Tomtec) and incorporated radioactivity was measured using a Beta Plate reader (Packard). Stimulation index (SI) was determined as:

SI = experimental count / count in wells with MRC-5 control antigen.

SI ≥3 and a Δ experimental count (count in the presence of pp65 antigen - count in the presence of control MRC-5 antigen) of 500 were considered indicative of a positive response to pp65. The highest SI for each time point is given in Results.

3.10. *Study design of clinical trials*

Trial A. The vaccine study, conducted in Richmond, VA, enrolled 20 healthy HCMV-seronegative volunteers, 20–43 years of age. By use of a double-blind format, volunteers were randomized to receive either two intramuscular injections of $10^{6.8}$ $TCID_{50}$ ALVAC-CMV-gB or two intramuscular injections of $10^{6.8}$ $TCID_{50}$ of ALVAC-RG 1 month apart from each other. Both groups received a single subcutaneous dose of $10^{3.5}$ pfu of Towne vaccine 90 days after the initial dose of ALVAC vaccine. Samples of blood, urine, and saliva were collected for each subject on days 30, 60, 90, 97, 114, 120, 180, 270, and 360 after immunization. Two subjects, both assigned to the ALVAC-RG group, withdrew from the study prior to completion, for reasons unrelated to study participation. The data on their immune responses were not included. All subjects were monitored for vaccine-associated illness: (1) the subjects maintained a temperature chart for 2 weeks after vaccination; (2) the subjects were contacted by phone by a research nurse 3 days after each vaccine administration; (3) the subjects self-reported (via a diary card) any acute local or systemic reaction, including any pain, swelling, or erythema; and (4) at follow-up visits, an interim history was obtained.

IgG antibodies to gB in sera were measured by an enzyme immunoassay at the Medical College of Virginia (Richmond, VA, USA), and we determined neutralizing antibodies by a standard reduction assay [39]. Urine and saliva samples were cultured, before

and after concentration, in duplicate on MRC-5 fibroblasts, and white blood cells were prepared from each plasma sample and cultured for HCMV. Groups were compared with repeated-measures analysis of variance assessing group versus time interactions. For antibody data, the log of the reciprocal titers was analyzed. A neutralizing titer of 1 was attributed to sera showing incomplete neutralization at the lowest dilution tested (1 : 4 or 1 : 8).

Trial B. This randomized, placebo-controlled clinical trial was conducted at The Children's Hospital of Philadelphia. Healthy adult volunteers, 18-35 years of age, were eligible to participate unless one or more of the following exclusion criteria was present: history of severe adverse reaction or allergy to any vaccine; known or suspected allergies to eggs, monosodium glutamate or neomycin; history of any immunosuppressive disease or major chronic disorder; receipt within the previous 6 months of immunoglobulin therapy, blood or blood products, steroids, oral or parenteral immunosuppressive therapy, or cimetidine; prescription medication usage (other than oral contraceptives); planned immunization with other vaccines within the first 7 months of the study period and pregnancy.

Two months before the first immunization, eligible volunteers were screened for HCMV antibodies, as well as hematological and biochemical status. Final inclusion into the study was offered to subjects without laboratory abnormality and with no detectable levels of HCMV neutralizing antibodies. Inclusion was also proposed to additional 5 subjects with high titers of HCMV neutralizing antibodies. At that time all subjects had 50 ml of blood drawn for preparation of Epstein-Barr virus-transformed lymphoblastoid cell lines, as well as HLA typing using standard molecular techniques (performed by the Immunogenetics Laboratory of the Children's Hospital of Philadelphia, PA., USA).

Two months later, the seronegative individuals were randomized to receive ALVAC-CMV-pp65 or placebo at a ratio of 2:1. The five subjects with high titers of HCMV neutralizing antibodies were all immunized with ALVAC-CMV-pp65. All subjects received 4 immunizations intramuscularly at month 0, 1, 3 and 6. In order to increase the dose of ALVAC administered, two injections were given concomitantly. Prior to each immunization, a negative pregnancy test was obtained for female participants. Subjects were seen monthly for the first 7 months after the first immunization and then at month 12. During each study visit blood was drawn for hematological, biochemical, and immunological tests. Immune

responses to HCMV-pp65 were determined in our laboratory at the Wistar Institute, Philadelphia. Specific immunologic assays were performed as follows: HCMV-pp65-specific lymphocyte proliferation at months 0, 3, 4, 6, 7, 12, and 26; HCMV-specific cytotoxic T cells (CTL) at months 0, 3, 4, 5, 7, 12, and 26 and ELISA antibodies to HCMV-pp65 at months 0, 3, 4, 6, 7, (Table 2).

All subjects were observed for 30 minutes after each immunization to detect immediate adverse effects. Telephone inquiries were made on days 2 and 8 after each immunization. In addition, subjects were required to maintain diaries that included a list of systemic reactions from day 0 to 7 and local reactions at day 3 after each dose. Interim medical histories were obtained at each study visit to detect unreported events. Adverse reactions were graded as follows: mild (Grade 1) - subject aware of symptom, but symptom is tolerated and does not interfere with daily activities; moderate (Grade 2) - symptom interferes with or restricts the subject's ability to perform usual activities; severe (Grade 3) - symptom is incapacitating and prevents the subject from working or carrying out usual activities.

Table 2. Study Timetable

	Month								
	0	1	3	4	5	6	7	12	26
Vaccination	+	+	+			+			
LP	+		+	+		+	+	+	+
CTL	+		+	+	+		+	+	+
Ab	+		+	+		+	+		

4. Results

4.1. Aim 1: *To determine the prevalence and characteristics of pp65-, pp150-, IE1-, gB- and pp28-specific CTL responses in a randomly selected racially and geographically divergent population of healthy, naturally HCMV seropositive individuals*

4.1.1. *CTL responses to pp65, IE1-exon4, gB, pp150, and pp28 in HCMV-seropositive donors*

Effector cells were restimulated by individual HCMV antigen–expressing canarypox recombinants. Lytic activity of restimulated memory CTLs was tested in antigen-specific ^{51}Cr-release assays. CTLs specific for pp65 and IE1-exon4 proteins were detected in 92% and 76% of the seropositive donors, respectively, whereas gB- and pp150-specific CTLs were identified in 33% and 30% of the donors tested, respectively; no pp28-specific lysis of target cells was detected in any individual [**I**/ Table 1]. The number of PBMC obtained from the blood of 4 donors (KL, H-P, H-47, and H-48) was sufficient for testing only pp65- or pp65- and IE1-specific CTLs; therefore, we cannot exclude the presence of CTLs specific for other HCMV antigens. Of the volunteers tested for 4 or 5 different HCMV antigens, 5 (TK, MT, H-35, H-36, DF) were positive for 4 antigens (pp65, IE1- exon4, gB, and pp150). Two donors (BK and H-33) were positive for 3 antigens (pp65, IE1-exon4, and gB or pp150); 9 donors showed double-CTL specificity (pp65 and gB in donor GE; pp65 and IE1-exon4 in donors H-6265, H-22, MBM, BJ, H-23, CK, KI, and H-49); and 6 donors showed single specificity (H-6226, H-34, H-12, NJ, MA, and BI). Of these latter 6 donors, 2 highly seropositive donors (H-12 and NJ) exhibited only IE1-exon4–specific CTLs; repeated testing of these donors (5 times over a 2-year period for donor H-12; 3 times over a 1-year period for donor NJ) consistently revealed IE1-exon4 CTL positivity, as well as pp65, pp150, and gB negativity. In addition, the profile of CTL specificity remained stable over a 27-month observation time, as exemplified in [**I** /Figure 2] for a highly seropositive individual (H-35) who had specific CTLs for IE1-exon4, pp65, gB, and pp150 (shown only for IE1-exon4 and pp65). The seronegative donors tested showed no CTL activity against any of the HCMV antigens.

4.1.2. *Strong pp65- and IE1-specific CTL activity of bulk cultures reflects similar pp65 and IE1 CTL precursor frequencies*

Limiting dilution assays were done to determine CTL precursor frequencies with PBMC of 2 donors who were repeatedly positive for both pp65 and IE1-exon4 in bulk cultures. [**I** /Figure 3] shows the results obtained with donor KI (NA titer, 1 : 512). Both the IE1-exon4 and pp65 antigens were detected at similarly high frequencies, that is, $566/10^6$ PBMC and $699/10^6$ PBMC, respectively. Precursor frequencies were also similar for the 2 antigens in donor DF (NA titer, 1 : 512), for whom IE1-exon4- and pp65-specific CTL precursor frequencies were $78/10^6$ PBMC and $67/10^6$ PBMC, respectively.

4.1.3. *HCMV-infected monocytes restimulate both IE1-exon4– and pp65-specific memory CTLs*

To test whether IE1-exon4 presented together with pp65 by the same stimulator cells can restimulate IE1-exon4–specific CTLs, we performed experiments by using effector cells restimulated with HCMV-infected monocytes, that is, cells that express HCMV IE antigens at very low levels [102, 28, 57]. Presentation of the input pp65 to CTLs has also been reported [104]. PBMC from IE1- and pp65-responder donor H-22 were restimulated for 8 days with autologous monocytes infected with the Towne strain of HCMV (MOI, 2–4), and effector cells were tested for IE1-exon4– and pp65-specific CTL activity on autologous and MHC-mismatched Vac-recombinant–infected B-LCL cells. Both IE1-exon4– and pp65-specific CTL activity was detected [**I** / Table 2], indicating that pp65 and IE1-exon4 proteins in monocytes can restimulate memory CTLs. Effector cells of the same donor restimulated with canarypox-pp65 or canarypox-IE1 infected B-LCL, lysed target cells coinfected with Vac-IE1 and Vac-pp65, indicating that expression of both antigens did not interfere with pp65- or IE1-specific CTL activity [**I** / Table 2].

4.1.4. *HLA restriction of effector populations*

It is generally accepted that activated T cells recognize antigens on the surface of antigen-presenting cells in the context of MHC molecules. To determine the MHC restriction of the stimulated CTL effectors, cells were tested against a panel of partially HLA-matched

and HLA-mismatched targets that express HCMV proteins. Analysis of selected CTL responders to identify restricting alleles for pp65-, IE1-exon4–, and pp150-specific CTLs indicated that pp65 was presented by A1, A2, A28, A68, B7, and B12/44 alleles; IE1-exon4 was presented by class I HLA alleles A1, A2, A3, B7, B8, B18, and B70; and pp150 was presented by the A3 and B14 alleles [**I** /Table 3]. Analysis of the restricting allele for gB-specific effector cells identified the class II antigens DR 7, DR15, and DQ 2 or 6 (not shown). [**I** / Figure 4] shows the results obtained with donor H-36, whose cells consistently generated high levels of CTL against pp65, IE1-exon4, and pp150. pp65- and IE1-exon4-specific CTLs of this donor were directed by the HLA-A2 molecule [**I** /Figure 4A and B], as only target cells sharing the A2 allele were lysed. The pp150 antigen was presented by the A3 allele [**I** /Figure 4C]. Analysis of effectors from donor H-36 on partially HLA-matched target cells of donors sharing alleles other than HLA-A2 or HLA-A3 revealed no pp65-, IE1-exon4–, or pp150-specific lysis of target cells (not shown). The percentage-specific lysis of class I HLA-matched targets was similar to the cytotoxic response against the autologous targets, which suggests a predominance of activated CD8+ cells specific for epitopes presented by the particular HLA allele.

4.1.5. *Phenotype of effector cells*

The phenotypic characteristics of the pp65-, IE1-exon4–, pp150-, and gB-specific effector cells were examined following depletion of CD4+ and/or CD8+ lymphocytes by use of the respective MABs plus complement. The subpopulations of the donor effector cells were tested against autologous and MHC-mismatched target cells that express HCMV antigens [**I**/Figure 5]. Depletion of both CD4+ and CD8+ cells completely abrogated killing by the pp65-specific effector cells of donor H-35 (not shown), whereas partial inhibition of specific lysis was observed when either CD8+ or CD4+ cells were depleted (21% and 32% inhibition, respectively; [**I** / Figure 5A]. The depletion experiment was repeated 3 times with PBMC of donor H-35 at different time points with similar results, indicating the consistent presence of both CD4+ and CD8+ pp65 effector cells in this donor. To exclude the possibility that the long restimulation *in vitro* and the addition of IL7 and IL2 favored the growth of CD4+ effector cells, we tested pp65-specific effector cells after a short (6 days) restimulation *in vitro*

without cytokines; phenotypic analysis again revealed mixed effector populations of CD4+ and CD8+ pp65-specific CTLs (data not shown). In contrast, phenotypic analysis of pp65-specific effector cells from another donor (H-36) showed that effector cells were CD8+ [**I**/Figure 5B]. The CTL response was exerted mainly through CD8+ cells specific for pp65 in an additional donor (H-33), pp150 (donor H-36), and IE1-exon4 (donors H-35 and H-36) [**I**/Table 4]. gB-specific effector cells, however, lost their activity after CD4+ depletion, but did not after CD8+ depletion (donor H-35) [**I** /Table 4]. The CD8+ phenotype of IE1-exon4 and pp150 CTLs and the CD4+ phenotype of gB-specific CTLs is consistent with the finding that IE1-exon4 and pp150 are presented by class I alleles, whereas gB is presented by class II alleles.

4.2. Aim 2: *To determine the tolerability and the immunogenicity of the ALVAC-CMV-gB recombinant in combination with the Towne vaccine in humans Trial A.*

Two groups of volunteers were randomly assigned to receive two injections, 1 month apart, of either ALVAC-CMV-gB (10 volunteers) or ALVAC-RG (8 volunteers). Both groups received a single injection of Towne vaccine 90 days after the first ALVAC injection.

4.2.1. *Safety and reactogenicity*

For the first two doses, reactogenicity was mild, and both ALVAC-CMV-gB and ALVAC-RG were well tolerated. Towne vaccine (dose 3) induced a frequency of local or systemic reactions similar to that for the ALVAC vaccines. No subject had Towne vaccine virus recovered in urine, saliva, or blood.

4.2.2 *Immunogenicity*

ALVAC-CMV(gB), while not eliciting detectable levels of antibodies itself, could prime an immune response to gB. After two injections of ALVAC and before booster with Towne on day 90, a low increase ($P<0.057$) in anti-gB ELISA titers was observed in ALVAC-CMV-gB recipients, whereas no change was observed in the neutralizing titers. No change in either anti-gB ELISA or HCMV-specific neutralizing antibodies was observed in ALVAC-RG recipients [**II** / Table 1].

After administration of the Towne vaccine on day 90, the ALVAC-CMV-gB group developed significantly higher mean ELISA titers against HCMV gB [F(9144)=4.03, P<0.0001] and mean neutralizing titers [F(9142)=8.9, p<0.0001] than the ALVAC-RG group. The kinetics of the responses of the two groups were also different: in the ALVAC-CMV-gB group, the ELISA anti-gB response increased as early as day 7 and the neutralizing response as early as day 14 after Towne booster, whereas 28 days were needed to obtain a response, measured by either ELISA or neutralizing titers, in the ALVAC-RG recipients. The titers also persisted at significantly higher levels up to day 360 in the ALVAC-CMV-gB group, compared with the ALVAC-RG group [**II** / Table 2].

Priming with ALVAC-CMV-gB allowed the Towne vaccine to induce peak ELISA titers to gB and HCMV neutralizing titers at levels significantly higher than those observed among naturally seropositive subjects. For the naturally seropositive subjects, the geometric mean ELISA antibody titer to gB was 1 : 25591, compared with a peak (180 days) geometric mean titer of 1 : 89321 for the subjects primed with ALVAC-CMV-gB (P= 0.03). The same was true for neutralizing titers. Naturally seropositive subjects had a geometric mean neutralizing titer of 1 :79, compared with a peak (180 days) geometric mean titer of 1 : 259 for the subjects primed with ALVAC-CMV-gB (P = 0.008).

4.3. Aim 3: ***To determine whether an ALVAC-CMV-pp65 recombinant vaccine is well tolerated in humans and whether this recombinant induces long lasting pp65-specific CTL, helper T cell responses and antibodies in a heterologous HCMV seronegative population comparable to that of naturally seropositive individuals Trial B.***

A total of 27 volunteers were enrolled in the trial. Twenty-three of them were seronegative for HCMV and received the vaccine (14) or placebo (9) and 4 were seropositive. Two seronegative canarypox-CMV-pp65 immunized subjects did not complete the study, one of whom received an initial dose, while the other received two doses. The purpose in immunizing highly seropositive volunteers was primarily to assess the safety of the vaccine in this population.

4.3.1. *Reactogenicity*

Elicited reactions [**III** / Table 1], as recorded on diary cards, were more common in subjects who received vaccine than in placebo recipients. Most were of mild to moderate severity and occurring typically during the first few days post-vaccination. There was no evidence that either the severity of the reaction or the frequency of occurrence increased after administration of successive doses. Reactogenicity did not appear greater in the four seropositive subjects than in the seronegative vaccine recipients.

Among the local reactions, most consisted of mild to moderate pain or tenderness. Headache, malaise, nausea, and myalgia were the most commonly reported systemic reactions.

Only two reported adverse events were considered as related to vaccination (both among seronegative canarypox-CMV-pp65 recipients): a severe local reaction post-dose two (subject was discontinued from the study), and 35 days of rash ("contact dermatitis") post-dose three in a second subject.

4.3.2. *pp65-specific CTL responses in all of the originally seronegative vaccinees*

PBMC obtained from the vaccinees at months 0, 3, 4, 5, 7, 12, and 26 were tested for pp65-specific CTL activity in bulk cultures. The HCMV seronegative individuals were CTL-negative at time 0 and the placebo recipients remained CTL-negative, whereas all individuals who received the canarypox-CMV-pp65 vaccine responded with pp65-specific CTL activity at each time point tested [**III** / Figure 1]. All of the eight seronegative vaccinees tested after 2 doses of vaccine exhibited HLA class I-restricted pp65-specific CTL activity. After the third inoculation, all of the 14 originally seronegative vaccinees showed CTL activity when tested at months 4, 5, 7, 12 and all of the 5 tested were positive at M.26. The 4 naturally seropositive subjects were pp65-specific CTL-positive at time 0 and remained positive for the entire study period. Figure 2 shows the pp65-specific CTL activity of a representative, originally HCMV seronegative individual (vaccinee 19) at month 5 (2 months after the second immunization) and month 12 (6 months after the third immunization). Specific lysis was obtained even at very low E:T ratios (3:1 at month 5 after the third, and 1.5:1 at month 12 after the fourth immunization, respectively). Specific lysis at similarly low E:T ratios was observed with the majority of vaccinees (not shown). Table 3 summarizes the HLA haplotypes of the 14 originally seronegative and 4 seropositive vaccinees. 17 of whom exhibited one or more HLA

alleles (A1, A2, A3, A68, B7, B35) previously shown to present pp65 epitopes. All of these individuals mounted pp65-specific CTL after vaccination. These results demonstrated that HCMV-pp65, as expressed by the ALVAC recombinant, induced CTL responses in individuals with different HLA haplotypes, that two doses of canarypox-CMV-pp65 were sufficient for CTL induction, and that CTL activity did not change significantly during the 20 months following the last vaccine dose.

4.3.3. *Similar pp65-specific CTL precursor frequencies in canarypox-CMV-pp65 vaccinees and naturally seropositive individuals*

CTL precursor frequencies (CTLp) were determined at month 7 or 12 (1 and 6 months after the fourth inoculation) in 4 originally seronegative volunteers who received the canarypox-CMV-pp65 vaccine and in 2 naturally seropositive donors who were not vaccinated. In 3 of the 4 vaccinees, the CTLp/10^6 PBMC was similar to that of the 2 naturally seropositive donors (range 105-257 for vaccinees 27, 15, and 29 versus 155 and 206 for naturally seropositives), whereas in the fourth it was lower at 61 [**III** / Table 2]. No CTLp were detectable in volunteer 25, who was a seronegative subject inoculated with placebo. In an experiment to test the number of CTLp in the course of the immunization process, PBMC cryopreserved at 0, 4, and 7 months from vaccinee 35 were used as effector cells. CTLp were not detectable at month 0, not tested after 1 or 2 doses, but were detected after 3 (92/10^6 PBMC at month 4), and after 4 vaccinations (264/10^6 PBMC at month 7) (Figure 3). These results indicate that the number of memory CTL increased during the repeated immunization of this individual with canarypox-CMV-pp65. Furthermore, CTLp detected at 7-12 months after the first immunization with the canarypox-CMV-pp65 vaccine in seronegative individuals were similar to that detected in HCMV seropositives after natural infection that occurred at an undefined time in the past and which may have been boosted or expanded by multiple exposures.

4.3.4. *Phenotype of pp65-specific CTL*

The phenotype of pp65-specific CTL was determined at month 5 in originally seronegative individuals who received the canarypox-CMV-pp65 vaccine. [**III** / Figure 2] shows the results of a depletion experiment using cells from vaccinee 15. While depletion of

effector cells with anti-CD4 antibodies and C' did not change the percent lysis of the target cells, depletion with anti-CD8 antibodies and C' or with a mixture of anti-CD4 and anti-CD8 antibodies and C' decreased the lysis to <10%, indicating that the effectors were CD8+. Of 13 vaccinees analyzed for phenotype of pp65-specific CTL, 11 exhibited CD8+ effector cells. In two vaccinees the determination of CTL phenotypes did not give clear results because of low pp65-specific CTL activity or the apparent presence of effector cells partially resistant to CD4 and CD8 antibodies and C'.

Table 3 HLA haplotype of the canarypox-CMV-pp65-vaccinated volunteers.

Subject	pp65-specific CTL	HLA alleles[a]							
		A	A	B	B	DRB1	DRB1	DQB1	DQB1
15	+	**02**	**03**	37	22	0701	1104	0202	0301
9	+	**02**	**68**	1801	52	0404	15	0302	0601
16	+	**01**	**03**	0801	**35**	0101	0401	0501	0301
8	+	**02**	31	4402	**35**	0403	1301	0305	0603
2	+	**02**	25	15	4402	0401	0701	0301	03032
5	+	**02**	**68**	**07**	58	1102	1201	0301	0501
19	+	**0101**	24	**35**	3701	1001	0404	0501	0302
10	+	**01**	**02**	0801	35	03011	1305	0201	0301
7	+	**02**	**03**	**07**	15	15	0401	0602	03
22	+	**01**	**02**	08	44	15	0407	06	0301
27	+	32	11	44	**35**	11	11	0301	03
29	+	**01**		**07**	08	0701	03011	02	03
30	+	02	03	**07**	1401	0701	13	02	06
32	+	**02**	33	**07**	1503	11	12	0301	05
31	+	31		4901	15	01	11	05	0301
33	+	29	11	**07**	44	1501		06	
35	+	**01**	**02**	08	44	0301	1501	0201	0602
38	+	**01**	**68**	08	53	1302	1401	0503	0604

[a] Alleles known to present pp65 epitopes are printed in bold.

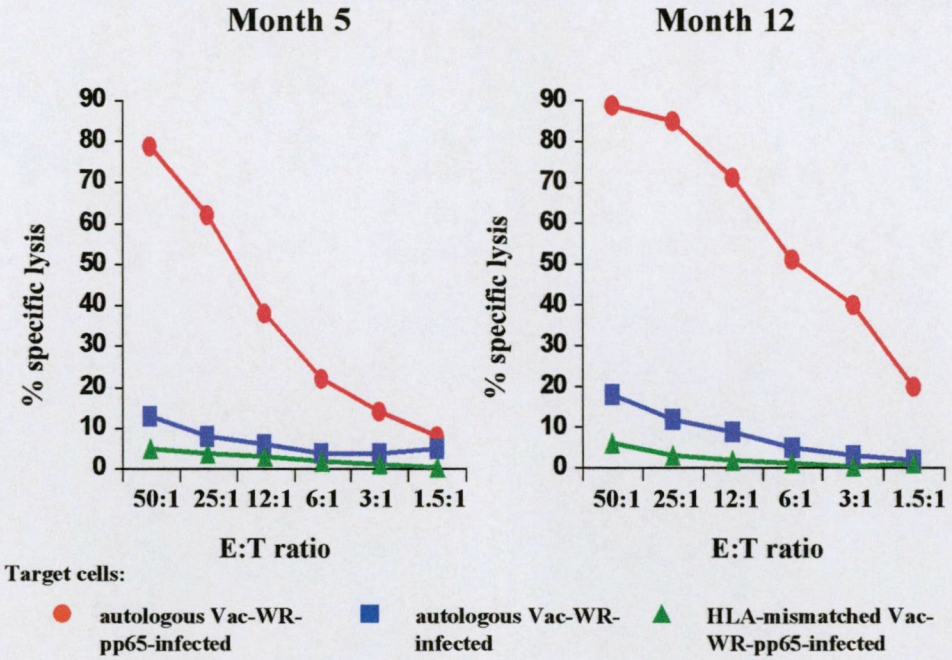


Fig. 2. pp65-specific CTL activity of PBMC from vaccinee 19 at 5 and 12 months after initial immunization. PBMC were restimulated with canarypox-CMV-pp65 *in vitro* for 12 days. Cytolytic activity of effector cells was measured in 4-h ^{51}Cr-release assays using autologous and HLA-mismatched EBV-transformed B lymphoblastoid cell lines as target cells.

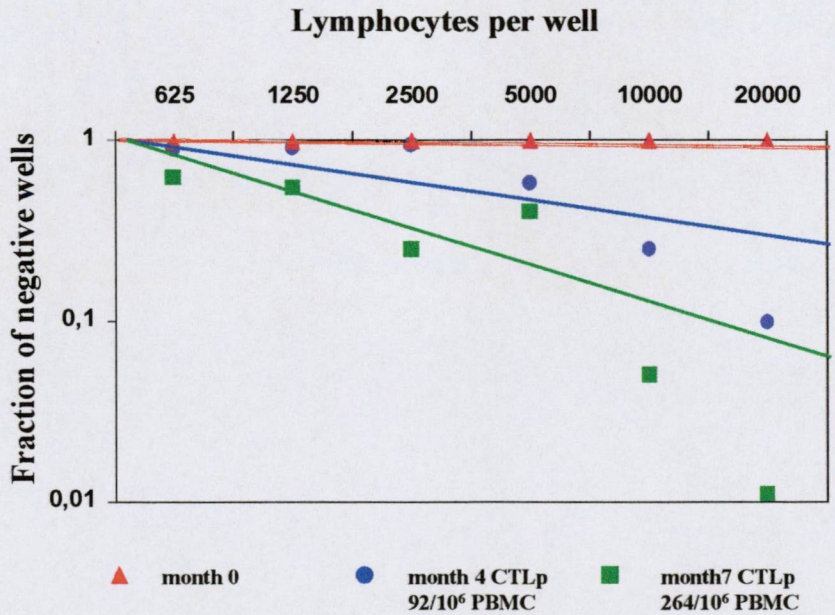


Fig. 3. pp65-specific CTL precursor frequency of an originally seronegative donor vaccinated with the canarypox-CMV-pp65 (donor 35). PBMC cryopreserved at 0, 4, and 7 months were used in limiting dilution assay.

4.3.5. *pp65-specific lymphoproliferative responses in vaccinees*

To determine whether pp65 as expressed by canarypox-CMV-pp65 elicits helper T lymphocytes, pp65-specific lymphocyte proliferation responses were analyzed. Lymphoproliferative responses to a purified pp65 preparation were measured for all volunteers [**III** / Table 3]. In the originally seronegative individuals who received the canarypox-CMV-pp65 vaccine, pp65-specific responses were negative (SI<3) at the time of the first immunization, but became positive (SI≥3; mean SI = 11.04) after two immunizations. All of the vaccinated individuals showed pp65-specific lymphocyte proliferation, 7 were positive at all time points tested and 7 were positive at two to four time points (M.3 to M.12). Four of the five vaccinated volunteers tested at month 26 were positive [**III** / Table 3].
Originally seronegative individuals who received the placebo remained negative. Originally seropositive vaccinees remained positive, with some decrease in lymphocyte proliferation indices after three inoculations. However, by month 12, stimulation indices were greater than before the immunization process [**III** / Table 3].

4.3.6. *Antibody responses in vaccinees*

Antibodies were measured by a pp65-specific ELISA in a total of 21 volunteers at different time points after the first vaccination. The originally seronegative vaccinees who received the canarypox-CMV-pp65 vaccine developed pp65-specific binding antibodies (mean OD 0.077 - 1.261) after 2 or 3 inoculations and remained positive during the observation period [**III** / Table 4]. Originally seronegative vaccinees who were inoculated with placebo remained negative.

5. Discussion

5.1. We analyzed the HCMV-specific CTL responses of naturally seropositive healthy adult blood donors, with a variety of HLA haplotypes, to determine the prevalence of CTLs specific to various HCMV proteins. *In vitro* cytotoxicity assays were used, which, to date, remain the best way to examine antigen-stimulated T cells that function as CTLs. All 26 HCMV-seropositive donors mounted specific CTL responses against at least 1 of the HCMV proteins, pp65 or IE1-exon4. In addition, PBMC of about one third of the seropositive donors revealed CTLs specific to the pp150 and/or gB proteins. However, no pp28-specific CTLs were evidenced, indicating that this protein is not among the immunodominant CTL targets induced by natural HCMV infection. No obvious differences were observed among racial groups of different geographic areas in the cytotoxic pattern of response to the specific antigens. On the other hand, CTL responses did correlate with HCMV NA levels, that is, donors with HCMV NA titers >1 : 128 were more likely to have CTLs positive for multiple HCMV antigens, whereas all but 2 of the moderately seropositive donors (HCMV NA titers <1 : 96) had CTLs specific for only pp65 or for pp65 and IE1-exon4 (**I./** Table 1), indicating the immunodominance of these 2 antigens. These results also suggest that high NA titers are indicative of a broad spectrum of CTL targets in seropositive donors.

Previous reports have implicated the most abundant protein constituents of the HCMV virion, including the major matrix proteins pp65 and pp150, in CTL induction [104]. The pp65 was identified as the immunodominant CTL target [103, 66]. CTLs have also been shown to recognize exogenously introduced pp65, suggesting that the pp65-specific CTL response acts on virus-infected cells soon after infection and that recognition and cell lysis occur before the onset of viral replication and virion assembly [104]. In a study to determine pp65-specific CTL precursor frequencies, Wills et al. [130] found that all 7 donors tested had CTLs specific for the pp65 protein, and a very high proportion of all HCMV-specific CTLs in any given donor was specific for this single virus protein. In our study, 24 of the 26 tested seropositive donors responded with pp65-specific CTLs on *in vitro* pp65-specific stimulation.

The ubiquity of pp65-specific CTLs in natural HCMV infection suggests that pp65 epitopes can be presented by a wide variety of HLA alleles. Indeed, HLA-B35 [66], A2, B7,

B8, and B35 [130] were identified as pp65 epitope-presenting alleles. In our panel we confirmed and identified alleles A1, A2, A28, A68, B7, and B12/44 presenting pp65.

The pp65-specific effector cells of donor H-35 comprised a mixed population of CD4+ and CD8+ cells, in contrast to the exclusively CD8+ pp65-specific CTLs reported in the literature. The discrepancy might reflect the use of EBV-transformed B lymphoblastoid cells that express both MHC class I and II antigens, while other investigators used mainly fibroblast target cells that express MHC class I alleles only, which do not enable detection of CD4+ effector cells. We also found donor variability in the phenotype of pp65-specific CTLs, since donors H-36 and H-33 showed only CD8+ effector cells. The proportion of pp65-specific CTL responders with both CD4+ and CD8+ effector cells is not known. Although the role of CD8+ CTLs in prevention of HCMV disease is well known, the significance of CD4+ effector cells remains unclear. CD4+ CTLs may be effective against latently infected cells with express HLA–class II alleles.

Studies in the murine CMV (MCMV) model have shown the important contribution of IE1 proteins to MCMV immunity and protection against the disease, and an antigenic nonapeptide has been mapped to IE1. Vaccination of mice with the recombinant vaccinia virus that encoded a chimeric protein carrying the optimal 9-amino-acid IE1 epitope sequence elicited CD8+ T lymphocytes with antiviral activity and, furthermore, protected against lethal disease. [94-96, 45]. In an earlier study, a high proportion of HCMV-specific CTLs recognized IE1 in 2 donors tested [14]. IE1-specific CTLs were detected in bulk culture assays [46, 104], but only in 3 and 2 donors respectively, and IE1 CTLs were less dominant than pp65 CTLs. Split-well LDA revealed CTLs directed to the IE1 protein in 3 of 5 donors tested, but with significantly lower precursor frequency than that of pp65 CTLs [130]. In a recent study, the frequencies of CD8+ T cells that were directed against IE1 were similar to those directed against pp65 in donors tested by a flow cytometric assay [51]. In our study, IE1-exon4 -specific CTLs were nearly as prevalent as pp65-specific CTLs, and had similar precursor frequencies. Moreover, this response was maintained over a prolonged period of time, as shown in 10 donors, suggesting that detectability of IE1-exon4–specific CTLs is not a random phenomenon due to a reactivation episode or other reasons in some donors, but instead, that IE1-exon4–specific memory CTLs are constantly present in the circulation at detectable levels. The broad spectrum of HLA alleles that present IE1-exon4 (A1, A2, A3, B7,

B8, B18, and B70) also supports the high prevalence of IE1-exon4–specific CTLs. Gilbert et al. [35] reported that pp65 diminishes the IE1-specific CTL activity exerted by IE1-specific CTL clones through a selective abrogation of IE1-peptide presentation by pp65 and its associated serine/threonine kinase activity. In our study, IE1-specific effector cells restimulated with ALVAC-IE1-exon4 readily lysed target cells coinfected with Vac-pp65 and Vac-IE1. Similarly, we did not observe this inhibition in bulk CTL assays where HCMV-infected autologous monocytes were used to restimulate memory CTLs, a situation perhaps more relevant to the *in vivo* milieu. During HCMV reactivation, IE1 appears earlier than pp65, so IE1-exon4–specific CTLs may be more effective than pp65-specific CTLs for preventing reactivation. It is not clear why donors H-12 and NJ were negative for pp65-specific CTLs, although both donors have HLA class I alleles (A1 and B7, respectively) that presented pp65 CTL epitopes in other donors. Perhaps HLA A1 and B7 subtypes not identified in this study are involved.

Although 2 of the recently identified 10 CTL epitopes were outside exon4 [51], inclusion of the full-length IE1 protein in an HCMV vaccine may be problematic because of the known transactivating ability of HCMV-IE1 proteins [53]. Immunofluorescence analysis to localize the IE1 protein expressed in several forms by a panel of poxvirus recombinants revealed the full-length IE1 gene product predominantly in the nucleus with some cytoplasmic staining, whereas the exon4 protein was found only in the cytoplasm (unpublished observations). IE1-exons2 and 3, but not exon4, are required for binding to p107, a member of a family of cell-cycle inhibitors [48], and deletion of exon3 eliminates the transactivating ability of IE1 [74]. Taken together, these observations suggest that expression of only the exon4 segment of IE1 eliminates the cellular activation potential associated with IE1 while maintaining most of the CTL epitopes.

gB-specific CTLs were shown in a considerable percentage (33%) of seropositive donors, consistent with previous reports [14, 61]. Hopkins et al. [46] showed gB-specific CTLs in 4 out of 7 donors and found that these CTLs were MHC class II–restricted CD4+ lymphocytes. CD8+ MHC class I–restricted gB-specific CTLs have also been described [103, 104]. In donor H-35, having gB-specific CTLs in our study, the effector cells were found to be CD4+ lymphocytes, and MHC–class II molecules were identified as the restricting alleles.

Thus, gB protein can serve not only as an important inducer of NAs in the majority of the population but also as a target of CTL responses in about one-third of the population.

The pp150 protein has also been reported as an immunodominant target for HCMV-specific CTLs [103, 66, 13]. In our studies pp150-specific CTLs were detected in only 30% of seropositive donors, with A3 and B14 alleles presenting pp150 to CD8+ cells, being less dominant than pp65 or the IE1-exon4 protein.

The lack of detectable pp28-specific CTLs in our study might rest in the absence of donors with haplotypes presenting pp28 CTL alleles. Although vaccinia- and canarypox-HCMV recombinants that express pp65, pp150, IE1-exon4, and gB were constructed similarly, we cannot exclude the possibility that processing and presentation of pp28 was not authentic in cells infected with vaccinia- or canarypox-pp28, resulting in pp28-specific negativity in the CTL assays.

5.2. An important observation made in trial A is that the ALVAC-CMV-gB vaccine could prime for an immune response to a live, attenuated vaccine (Towne) in seronegative volunteers and induced peak ELISA anti-gB titers and neutralizing titers at levels equal to or higher than those observed among the naturally seropositive subjects.

Like other ALVAC recombinants tested in humans, the ALVAC-CMV-gB vaccine was well tolerated: only mild local reactions and minimal systemic reactions were observed. These reactions were similar to those observed with the HCMV Towne vaccine.

For HCMV, the majority of the immunogenic neutralizing epitopes are located on the gB envelope glycoprotein of the viral particle [15], and the data of the current study are consistent with this, as both antibodies to HCMV gB and neutralizing activity were boosted to high levels after priming with HCMV gB.

The very low levels of gB-specific IgG and neutralizing activities induced by ALVAC-HCMV-gB alone in humans were unexpected, because this vaccine induces detectable levels of these activities in mice and guinea pigs [37]. Mammalian cells support only an abortive infection by canarypox, with viral gene expression limited to only early genes and for a relatively short duration. As there is no evidence that expression of HCMV gB is lower in humans than in small mammals, HCMV gB may be a poorer immunogen in humans than in laboratory animals.

Results of this trial confirm similar trials using ALVAC expressing HIV-1 MN gp160 [21]: ALVAC alone induces a weak antibody response but primes for subsequent exposure to HIV envelope.

The ability of the Towne strain of HCMV to prevent the child-to-mother transmission of HCMV was previously evaluated [2]. Women who were naturally seropositive appeared to be protected from acquiring a HCMV infection, but women who received the Towne vaccine were unprotected. In this previous trial, the Towne vaccine was used at a very low dose and produced neutralizing titers that were 10- to 20-fold lower than those produced by a wild-type infection. Furthermore, it was observed that naturally seropositive adults and those with vaccine-induced immunity who had serum neutralizing titers >1:64 also had detectable levels of IgG antibodies to HCMV gB in nasal washes and saliva [124]. Because gB contains the majority of neutralizing epitopes, it was proposed that serum neutralizing titers >1:64 would be necessary for protection against wildtype infection [124]. The current trial has demonstrated the feasibility of using low doses of ALVAC-CMV-gB to prime the immune system to the gB protein and obtain enhanced antibody responses and neutralizing titers >1:64. Based on these results and the experience with the ALVAC-HIV glycoprotein recombinant [21], another trial to test the prime-boost protocol has been performed. 105 healthy HCMV-seronegative adults received either three doses of subunit gB with the adjuvant MF-59, or two doses of ALVAC-CMV-gB followed by two doses of subunit gB and adjuvant, or three doses of both vaccines administered concomitantly. All three immunization strategies induced high levels of neutralizing antibody and cell mediated immunity tested with lymphocyte proliferation and production of γ interferon, but after completion of the immunization regimen, no benefit of ALVAC-gB used as a primer to subunit gB could be demonstrated (data not published). It remains to be determined if the prime and boost strategy would induce a better CTL response.

5.3. In trial B, we show for the first time that pp65-specific CTL can be induced in humans by a recombinant vaccine candidate. Similarly to all other ALVAC recombinant vaccines tested so far in human volunteers the ALVAC-CMV-pp65 construct was safe and well tolerated. As also shown above HCMV-pp65 is a major CTL target in naturally seropositive individuals, and CTL induction in humans was demonstrated following Towne attenuated

virus vaccination in three of four immunized volunteers for 6 months [1], although the target protein(s) specificity of these CTLs was not determined. ALVAC-CMV-pp65 induced CD8+ CTL responses in all of the originally negative individuals, the responses were detectable at each time point included during a 1-year observation period. Moreover, the CTL precursor frequency was comparable to that of naturally HCMV-seropositive donors, and CTL responses were accompanied by HCMV-pp65-specific lymphocyte proliferation and antibody responses. All volunteers who were tested after the second of four inoculations were already positive for pp65-specific CTL, suggesting that two immunizations may be sufficient for induction of a CTL response. The possibility that the pp65-specific CTL responses of the seronegatives reflected natural intercurrent HCMV infection during the study period was excluded by the absence of HCMV gB-specific ELISA antibodies in these individuals (not shown), confirming the specificity of the pp65-specific immune responses.

The pp65-specific lysis of target cells in bulk CTL assays was observed at low E:T ratios ($\leq$6:1) in the majority of the volunteers at each time point, suggesting that pp65 is a strong CTL inducer in humans when expressed by the ALVAC-CMV-pp65 recombinant and that CTL responses do not differ significantly among individuals with different HLA haplotype. The HLA haplotype (Table 3) of the vaccinees was determined before the trial in order to guide selection of mismatched targets in the CTL assays, but vaccinees were not selected for the known HLA haplotypes presenting pp65. 13 of the 14 ALVAC-pp65 vaccinees exhibited one or more HLA alleles previously shown to present pp65 epitopes [66, 130].

Restriction maps of DNA extracted from laboratory strains and fresh isolates of human CMV show that all strains are distinct but related [19, 40, 108, 127, 128]. The significance of the restriction pattern polymorphism of the different strains is not clear, although genotypes characterized by specific sequence variations of the gB gene have been suggested to be associated with certain HCMV-diseases [32, 106, 122, 93, 20]. The pp65 gene of the ALVAC-CMV-pp65 recombinant was derived from the Towne strain of HCMV, and it is not proven that the pp65-specific CTL induced by the ALVAC-CMV-pp65 recombinant would recognize target cells expressing pp65 from other strains in naturally infected individuals. On the other hand, use of the same ALVAC-CMV-pp65 construct for restimulation of PBMC obtained from naturally seropositive donors in an *in vitro* CTL assay revealed high pp65-

specific CTL responses in 24 of 26 individuals tested, i.e., memory CTL cells from individuals of heterologous HLA haplotypes were restimulated by the ALVAC-CMV-pp65 recombinant, indicating common CTL epitopes in the Towne strain and the clinical strains that infected these individuals.

Our results do not provide direct evidence for the protective effect of ALVAC-CMV-pp65 against HCMV-diseases. However, the pp65 specific lysis at low E:T ratios in the bulk CTL assays and the similar CTL precursor frequencies of PBMC obtained from the vaccinees and naturally seropositive individuals suggest that immunization with ALVAC-CMV-pp65 might elicit sufficient immunological response to confer similar protection as is obtained through natural infection. Although the phenotype of pp65-specific CTL in some naturally seropositive individuals has been shown to be both CD8+ and CD4+, the phenotype of the CTL in 11 of the vaccinees was CD8+. Lymphocyte proliferation responses specific for pp65 detected in our *in vitro* assays indicate that the ALVAC-CMV-pp65 recombinant also stimulates CD4+ T cells, which suggests the release of cytokines that could contribute to the protective effect of the vaccine. Studies in murine models have shown that CD4 helper function, although not required for CD8+ CTL generation, is needed for persistence of CD8+ cell memory [105, 92].

The function of the anti-pp65-specific antibodies is uncertain but their induction by the ALVAC-CMV-pp65 recombinant reflects stimulation of B cells by the insert.

The following of our results are considered novel:

- In naturally seropositive individuals the HCMV IE1-exon4 protein is nearly as prevalent a CTL target as is pp65 with comparable precursor frequencies.
- No inhibition of IE1-specific CTL activity by pp65 was observed in CTL assays where HCMV-infected autologous monocytes were used to restimulate memory CTLs.
- The phenotype of pp65-specific CTL in one naturally seropositive individual was shown to be both CD8+ and CD4+.
- New antigen presenting alleles were determined for pp65 as A1, A28, A68, B12/44, for IE1-exon4 as A1, A2, A3, B18, B70, for pp150 as A3 and B14, and for gB as DR7, DR15, and DQ2 or 6.

- The ALVAC-CMV-gB vaccine could prime for an immune response to the live attenuated Towne vaccine and induced peak ELISA anti-gB titers and neutralizing titers at levels equal to or higher than those observed among naturally seropositive individuals.
- In vaccinated individuals HCMV pp65, as expressed by an ALVAC recombinant, was capable of inducing durable CD8+ CTL responses comparable to that of naturally HCMV-seropositive blood donors. Two doses of vaccine were sufficient for CTL induction. The CTL responses were accompanied by HCMV-pp65-specific lymphocyte proliferation and antibody responses.

These results may provide some insight into the immunology of HCMV infection and may contribute to the design of a vaccine that elicits effective CTL responses against both acutely infected cells, which express nonstructural IE1, and structural antigens, and latently infected cells during reactivation, in which nonstructural antigens are expressed earlier than structural proteins.

The priming effect of ALVAC-CMV-gB on antibody responses defines a vaccine strategy to induce potentially protective levels of neutralizing antibodies.

ALVAC-CMV-pp65 is the first recombinant vaccine capable of inducing HCMV-specific CTL responses in humans, suggesting the usefulness of this approach in the prevention of disease caused by HCMV infection.

Based on the above results the idea of a combined vaccine has been proposed, in which a canarypox recombinant containing several cytomegalovirus genes is used to generate cellular immunity effective against both acutely, and latently infected cells during reactivation, and to prime for augmented antibody responses to the viral glycoprotein administered as a subunit protein.

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8. Annex