I.

Cytotoxic T Lymphocyte (CTL) Responses to Human Cytomegalovirus pp65, IE1-Exon4, gB, pp150, and pp28 in Healthy Individuals: Reevaluation of Prevalence of IE1-Specific CTLs

Zsofia Gyulai,[1,4,a] Valeria Endresz,[4] Katalin Burian,[4] Steve Pincus,[3] Joseph Toldy,[4] William I. Cox,[3] Claude Meric,[5] Stanley Plotkin,[2] Eva Gönczöl,[1,4] and Klara Berencsi[1]

[1]*Wistar Institute, Philadelphia, and* [2]*Pasteur Mérieux Connaught, Swiftwater, Pennsylvania;* [3]*Virogenetics Corporation, Troy, New York;* [4]*Department of Medical Microbiology and South Hungarian Regional Blood Bank of National Transfusion Service, Albert Szent-Gyorgyi University, Szeged, Hungary;* [5]*Pasteur Mérieux Connaught France, Marcy l'Etoile, France*

The prevalence of human cytomegalovirus (HCMV) pp65-, pp150-, IE1-exon4–, gB- and pp28-specific cytotoxic T lymphocyte (CTL) responses was compared among 34 healthy individuals, grouped by neutralizing antibody titers. Moderately and highly seropositive donors showed predominantly pp65- and IE1-exon4–specific CTL responses (92% and 76% of the donors, respectively), with similar precursor frequencies in the 2 donors tested. 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 target in the CTL assay. Phenotypic analysis revealed a mixed effector population of $CD4^+$ and $CD8^+$ (1 donor) or only $CD8^+$ cells for pp65-specific effectors (2 donors). IE1-exon4– and pp150-specific effectors were $CD8^+$ (2 donors and 1 donor, respectively), whereas gB-specific CTLs were $CD4^+$ (1 donor). These data may help to design a cellular immunity-based vaccine effective against HCMV diseases.

Human cytomegalovirus (HCMV) is a member of the herpesvirus family and infection is widespread in the population. Although primary HCMV infection is often asymptomatic, infection or reactivation in the immunocompromised host is associated with significant morbidity and mortality. In allogeneic bone marrow transplant recipients, HCMV infection is the principal cause of death [1, 2]. HCMV is also the most common congenital or intrauterine viral infection, with an incidence between 0.2% and 2% of live births [3]. Studies in immunosuppressed humans have pointed to the importance of the cellular immune response, particularly CTLs, for controlling HCMV disease [4, 5], and recovery of HCMV-specific CTL responses in bone marrow and renal transplant recipients correlates directly with protection from CMV disease [6–9]. Thus, an effective HCMV vaccine will probably need to induce a specific CTL response.

Studies to identify which HCMV antigens might be important for immunity have shown that the lower matrix protein pp65 is the immunodominant target for HCMV-specific CTLs [8, 10–13]. Indeed, high pp65-specific CTL precursor frequencies were shown in seropositive donors [12, 13]. Epitope specificity of pp65-specific CTLs and the presenting HLA alleles were also identified in some donors [11, 12, 14]. HCMV IE1-, pp150-, gB-, and/or gH-specific CTLs have also been detected in studies, but the data are scarce, and the prevalence of protein-specific CTLs remains controversial [8, 12, 13, 15–20]. To our knowledge, the only study to examine CTL responses specific to pp28, which is a potent inducer of antibody production [21], reported the absence of these CTLs in all 4 seropositive donors tested [13]. At least some of the conflicting results might rest in the haplotype differences that affect antigen presentation in different racial groups and in the dominance of CTL epitope variations in HCMV strains in one geographical area but not in another.

In the present study, we compared the prevalence of IE1-exon4–, pp65-, pp150-, gB- and pp28-specific CTLs in a randomly selected population of 34 donors, representing a large panel of naturally seropositive, racially and geographically divergent, HLA-typed donors. We were especially interested in the nonstructural IE1-exon4 protein, which is abundantly expressed very early after infection (earlier than pp65) and con-

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Informed consent was obtained from the donors, and human experimentation guidelines of the Wistar Institute and of the Albert Szent-Gyorgyi University were followed.

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[a] Present affiliation: Department of Medical Microbiology, Albert Szent-Gyorgyi University, Szeged, Hungary.

Reprints or correspondence: Klara Berencsi, Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104 (Berencsi@wistar.upenn.edu).

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Table 1. Spectrum of human cytomegalovirus (HCMV) protein-specific cytotoxic T lymphocyte (CTL) activity of 34 donors.

Group and donor code[a]	Race	Neutralizing antibody (NA) titer	HLA haplotype				CTL activity[b]				
			Class I		Class II						
			A	B	DR	DQ	pp65	IE1-exon4	gB	pp150	pp28
Seronegative											
H-26	W	<1 : 8	1, 3	7, 8			−	−	−	−	−
H-62	W	<1 : 8	24/9, 25/10	51/5, 37			−	−	−	−	−
H-61	B	<1 : 8	26/10, 31/19	13, 35			−	−	−	−	−
H-40	B	<1 : 8	NT				−	−	−	−	−
H-27	B	<1 : 8	1, 17	8			−	−	−	−	−
K	W	<1 : 8	2	8, 27			−	−	−	−	−
PVL	W	<1 : 8	3, 10/26	7			−	−	−	−	NT
GG	W	<1 : 8	2, 3	7, 13	2/15, 10	1	−	−	−	−	NT
Moderately seropositive											
H-6226	W	1 : 12	3, 9/24	12, 18			+	−	−	−	−
KL	W	1 : 32	2, 11	8, 18			+	−	NT	NT	NT
H-6265	W	1 : 64	NT				+	+	−	−	−
H-34	B	1 : 64	24/9, 11	7, 62/15			+	−	−	−	−
H-22	W	1 : 64	3, 24	7, 8	3, 15	2, 6	+	+	−	−	−
MA	W	1 : 64	9/24, 19/32	15/62, 27	2/16, 4	1, 3	+	−	−	NT	−
MBM	W	1 : 64	3, 19/29	7, 12/44			+	+	−	−	NT
TK	W	1 : 64	3, 19/29	12/44, 14			+	+	+	+	NT
BJ	W	1 : 96	1, 2	8, 17/57			+	+	−	−	NT
BK	W	1 : 96	2	35, 40/60	4		+	+	−	+	−
Highly seropositive											
H-23	B	1 : 128	NT				+	+	NT	−	−
H-12	B	1 : 128	1, 33	60, 65	1, 13	5, 6	−	+	−	−	−
H-33	B	1 : 128	30, 74	14, 70	11, 13	2, 6	+	+	+	−	−
H-P	A	1 : 128	NT				+	NT	NT	NT	NT
CK	W	1 : 128	2, 10/25	5/51, 18	5/11, 6/14		+	+	−	−	−
MT	A	1 : 128	9/24, 19/31	5/51, 40/61	9, 6/14		+	+	+	+	−
NJ	W	1 : 128	10/25, 19/29	7, 18	1, 10	1	−	+	−	−	−
GE	W	1 : 128	3, 32	8, 60	3, 7		+	−	+	−	−
H-35	B	1 : 256	68	7, 70	7, 17	2, 6	+	+	+	+	−
H-36	B	1 : 256	2, 3	58	4, 13	3, 5	+	+	+	+	−
BI	W	1 : 256	2, 28	35, 53	1, 5/11	1, 3	+	−	−	NT	−
H-48	B	1 : 348	NT				+	+	NT	NT	NT
H-47	B	1 : 348	NT				+	+	NT	NT	NT
H-49	B	1 : 512	28	51/5, 35			+	+	−	−	−
KI	W	1 : 512	9/24, 11	7, 18	3, 5/11	2, 3	+	+	−	−	−
DF	W	1 : 512	3, 28	14, 40/61	1, 5/11		+	+	+	+	−
% positive/tested							92	76	33	30	0

NOTE. Peripheral blood mononuclear cells were restimulated in vitro with canarypox-pp65, -IE1-exon4, -pp150, -gB, or -pp28. W, white; B, black; A, Asian; NT, not tested.

[a] Donor's code with the initial H and nos. or H-P indicates donor in the United States; code with any other capital letters indicates donor in Hungary.

[b] Positivity reflects ≥10% HCMV antigen-specific lysis at effector-to-target ratios ≤50 : 1.

tinues to be expressed throughout the replicative cycle, making it an attractive vaccine candidate antigen together with pp65 for CTL induction.

Materials and Methods

Study population. Thirty-four healthy adults enrolled in the blood donor program of the Wistar Institute (Philadelphia, PA) and the Albert Szent-Gyorgyi University (Szeged, Hungary) were studied. The group was composed of 12 blacks, 1 Chinese, 1 Japanese, and 20 whites (age range, 22–60 years). HCMV seropositivity was determined by microneutralization assay [22], where sera with a neutralizing antibody (NA) titer <12 were considered negative, with an NA titer of 1 : 12 to 1 : 96 were "moderately" seropositive, and with an NA titer ≥1 : 128 were "highly" seropositive. Seropositivity was confirmed by standard ELISA by use of purified HCMV as a coating antigen and lysates of uninfected MRC-5 cells as a control antigen. For each serum dilution, optical density (OD) values obtained in control antigen–coated wells were subtracted from those obtained in HCMV antigen–coated wells. Sera with HCMV-specific OD values ≥0.2 than the reference negative serum, at a dilution of 1 : 100, were considered positive. 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) testing, which were performed by the Immunology and Histocompatibility Laboratory of the Hospital of the University of Pennsylvania, Philadelphia, and by the Blood Transfusion Center at the Albert Szent-Gyorgyi University. Table 1 lists the characteristics of each donor.

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

The pp65 gene was PCR amplified by use of genomic HCMV DNA as a template (Towne strain) and cloned as described elsewhere [23]. The IE1-exon4 gene (AD169 strain) was amplified by PCR from the pJD083 plasmid [24]. The pp150 and pp28 genes were amplified from HCMV genomic DNA (Towne strain) by PCR.

For restimulation of CTL effector cells in vitro, canarypox recombinants that express individual HCMV proteins were used. The gB-expressing canarypox recombinant has been described elsewhere [25]. pp65, pp150, IE1-exon4, and pp28 canarypox 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 elsewhere [25].

The WR strain of vaccinia virus (Vac-WR) and Vac-WR–based recombinants (Vac-gB, Vac-pp65, Vac-IE1, and Vac-pp28) that encode HCMV proteins were used for infection of target cells in cytotoxicity assays. The WR-gB recombinant (Vac-gB) was derived in the WR L variant vaccinia virus by use of previously described methods [26, 27]. WR-pp65, IE1-exon4, and pp28 recombinants (Vac-pp65, Vac-IE1, and Vac-pp28) were derived by use of a host-range selection system [27] 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 by use of specific monoclonal antibodies (MAbs) 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.

Target cell lines. Autologous B-lymphoblastoid cell lines (B-LCL) were established from each donor by incubation of 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 with 15% fetal calf serum (FCS) and were cryopreserved until use.

Effector cells. PBMC were separated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradients. Our preliminary results showed no difference between fresh or cryopreserved effector cells in their CTL activity; thus, fresh or cryopreserved PBMC were stimulated in vitro with autologous PBMC expressing HCMV proteins in the bulk CTL assays. Stimulator cells were prepared by infecting PBMC with canarypox virus vectors that encode HCMV pp65, IE1-exon4, gB, pp150, or pp28 at a multiplicity of infection (MOI) of 5. After a 1-h 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 3.5×10^6 cells/2 mL 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 m*M* L-glutamine (Sigma, St. Louis), 60 μg/mL gentamicin (Sigma), and 5 μ*M* 2-mercaptoethanol (Sigma). PBMC cultures were supplemented with 330 U/mL interleukin (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.

Restimulation of PBMC with HCMV-infected autologous monocytes. Monocytes were separated from PBMC by plastic adherence: 30×10^6 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 plaque-forming units per cell). After a 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 were tested after 8 days of in vitro restimulation by use of vaccinia recombinant–infected target cells.

Infection and labeling of target cells. Each of 5 individual HCMV protein–expressing vaccinia recombinants was used to infect 2×10^6–6×10^6 B-LCL (MOI, 10) for 1 h. Cells were diluted to 10^6/1.5 mL with culture medium and were incubated an additional 16 h, washed, and labeled with 100 μCi of ^{51}Cr-$NaCrO_4$ (Amersham, Arlington Heights, IL) for 1 h. Labeled cells were washed 3 times before assay.

Cytotoxicity assays. Cytolytic activity of antigen-driven effectors that are present in bulk cultures established from PBMC was measured in a 4-h ^{51}Cr-release assay. Nonadherent effector cells were collected, washed once, and plated in triplicate 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). Percentage-specific lysis was determined as follows: 100 × (experimental release-spontaneous release)/(maximal release-spontaneous release). For each target, spontaneous release was determined from wells containing medium only, and maximal release was calculated from wells containing 1% NP-40. Spontaneous release was always <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). Nonspecific background cytolysis was reduced by adding a 30-fold excess of unlabeled, WT-Vac–infected target cells to each well of the ^{51}Cr-release assay.

Limiting dilution assay. Fresh effector PBMC were plated at different cell numbers in 30 replicate wells and were cultured with γ-irradiated (3000 rad delivered by an MKA model 68A irradiator; Shepherd, Glendale, CA) autologous PBMC as feeder cells (6×10^4/PBMC per well) and with 4×10^3 autologous EBV-transformed lymphoblasts infected with Vac-pp65 or Vac-IE1 for 6–16 h, γ irradiated (3000 rad), and ultraviolet irradiated (10 min at a distance of 25 cm with a germicidal lamp) as stimulator cells. After 12–14 days, pp65- or IE1-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 [28]. ^{51}Cr-release in each well was considered significant at >3 SD above mean lysis observed in control wells (containing only feeder and stimulator cells).

Determination of CTL phenotype. Bulk CTL cultures were established from PBMC. After 8–13 days of culture in vitro, effector

cells were harvested, and aliquots of 3×10^6 cells were depleted of $CD4^+$ lymphocytes by anti-CD4 MAb + complement (C′), or of $CD8^+$ lymphocytes by anti-CD8 MAb + 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 (16 μg/mL). Nontoxic rabbit complement was from Accurate (Westbury, NY) and was used at a final dilution of 1 : 10. Effector cells were incubated with the antibodies at 4°C for 45 min. After washing with medium, cells were further incubated with complement for 30 min at 37°C, washed, and resuspended in the predepletion volume of medium so that nondepleted cells would remain at the original concentration, and CMV antigen-specific lysis was determined in a 4-h ^{51}Cr-release assay.

Statistical analysis. Linear regression analysis was done on results of limiting dilution assays (LDAs). Precursor frequency was determined by solving the equation of the best-fit line where the fraction of nonresponding wells equaled 0.37.

Results

CTL responses to pp65, IE1-exon4, gB, pp150, and pp28 in HCMV-seropositive donors. PBMC were either separated from fresh heparinized blood or reconstituted after cryopreservation, and 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. The seronegative donors tested showed no CTL activity against the HCMV antigens. 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 seropositive donors tested, respectively; no pp28-specific lysis of target cells was detected in any individual (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 CMV antigens other than pp65 and IE1. Of the volunteers tested for 4 or 5 different CMV antigens, 5 (TK, MT, H-35, H-36, DF) were positive for 4 antigens (pp65, IE1-exon4, gB, and pp150). As shown in figure 1, for 1 of these donors (H-36), the autologous target cells infected with Vac-pp65, Vac-IE1, Vac-gB, or Vac-pp150 showed significant lysis at E : T ratios of 40 : 1, whereas autologous WT-Vac–infected and HLA-mismatched (not shown) Vac-recombinant–infected target cells were not lysed, indicating that the cytotoxic response was MHC restricted. No CMV antigen-specific lysis of target cells was detected when PBMC from the same donor were restimulated in vitro with parental canarypox-infected autologous PBMC (not shown). 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

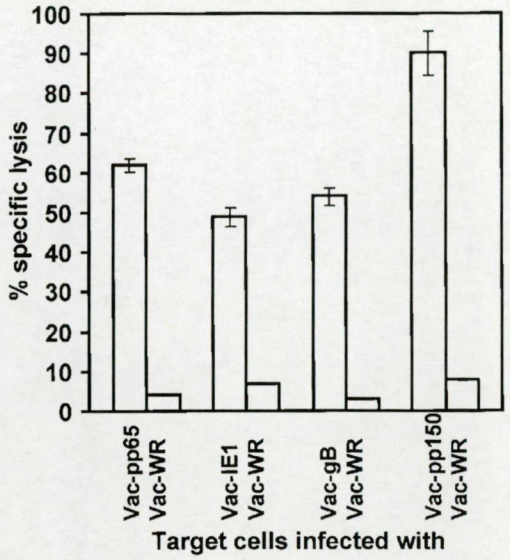

Figure 1. pp65-, IE1-, gB-, and pp150-specific cytotoxic T lymphocyte (CTL) activity of donor H-36. Cytomegalovirus antigen-specific CTL activity was measured in bulk cultures. Peripheral blood mononuclear cells (PBMC) were restimulated in vitro with canarypox recombinant-infected autologous PBMC for 11 days. Cytolytic activity of effectors was measured in 4-h ^{51}Cr-release assays by use of autologous and human lymphocyte antigen–mismatched Epstein-Barr virus–transformed B lymphoblastoid cell lines as target cells. Significant lysis of autologous target cells infected with Vac-pp65 or Vac-IE1, Vac-gB, or Vac-pp150 was observed at effector-to-target ratios of 40 : 1, whereas autologous WT-Vac–infected target cells were not lysed.

(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 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).

Strong pp65- and IE1-specific CTL activity of bulk cultures reflects similar pp65 and IE1 CTL precursor frequencies. LDAs 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. Figure 3 shows the results obtained with donor KI (NA titer, 1 : 512). Both the IE1-exon4 and pp65 antigens that were shown to be CTL targets in bulk assays were also detected at similarly high frequencies in LDAs, that is, $566/10^6$ PBMC and $699/10^6$ PBMC, respectively. Precursor frequencies were also similar for the 2 antigens in donor

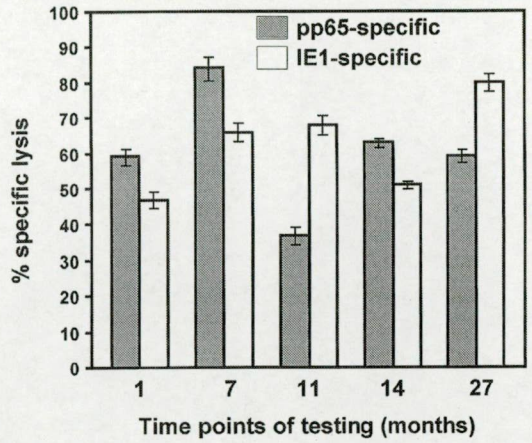

Figure 2. IE1- and pp65-specific cytotoxic T lymphocyte (CTL) activity of peripheral blood mononuclear cells (PBMC) of donor H-35 over a 27-month period. Cytomegalovirus IE1-exon4– and pp65-specific CTL were repeatedly measured in bulk cultures with PBMC from donor H-35. PBMC were restimulated in vitro with canarypox recombinant-infected autologous PBMC for 11–13 days. Cytolytic activity of effectors was measured in 4-h ^{51}Cr-release assays by using autologous and human lymphocyte antigen–mismatched Epstein-Barr virus–transformed B lymphoblastoid cell lines as targets. IE1-exon4– (□) and pp65- (■) specific lysis of target cells was observed at an effector-to-target ratio of 50 : 1. Antigen-specific lysis was calculated by subtracting percentage lysis of autologous WT-vaccinia–infected target cells from percentage lysis of autologous vaccinia recombinant-infected target cells.

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.

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 [29–31]. Presentation of the input pp65 to CTLs has also been reported [32]. 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. IE1-exon4– and pp65-specific CTL activity was detected (table 2), indicating that both pp65 and IE1-exon4 proteins in monocytes can restimulate CTL memory cells. Effector cells of the same donor restimulated with canarypox-pp65 or canarypox-IE1 lysed cells coinfected with Vac-IE1 and Vac-pp65, indicating that expression of both antigens did not interfere with pp65- or IE1-specific CTL activity (table 2).

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 CMV proteins. Figure 4 shows the results obtained from donor H-36, whose cells consistently generated high levels of CTL against pp65, IE1-exon4, and pp150. The pp65-specific CTLs of this donor were directed by the HLA-A2 molecule (figure 4*A*), because only target cells sharing the A2 allele were lysed. IE1-exon4 was also presented by HLA-A2, because only partially matched target cells sharing HLA-A2 were significantly lysed (figure 4*B*). The pp150 antigen was presented through the A3 allele (figure 4*C*). 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. Further analysis of selected CTL responders to identify other restricting alleles for pp65-, IE1-exon4–, and pp150-specific CTLs indi-

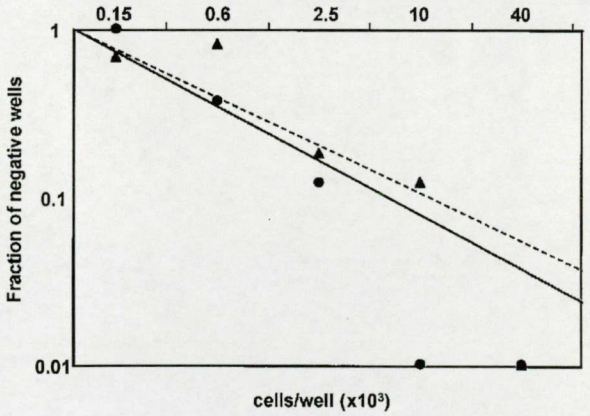

Figure 3. pp65- and IE1-specific cytotoxic T lymphocyte (CTL) precursor frequencies in donor KI. pp65- and IE1-specific CTL precursor frequencies were determined in limiting dilution assays (see Materials and Methods). Effector peripheral blood mononuclear cells (PBMC) were plated at different cell numbers per well and cultured with γ-irradiated autologous PBMC as feeder cells and autologous Epstein-Barr virus–transformed lymphoblasts infected with Vac-pp65 or Vac-IE1 as stimulator cells. After 12–14 days, pp65- or IE1-specific CTL activity in each well was determined on autologous Vac-pp65–infected or Vac-IE1–infected autologous WT-Vac–infected and human lymphocyte antigen–mismatched Vac-recombinant–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. ▲, Vac-IE1–infected target cells; ●, Vac-pp65–infected target cells.

Table 2. Human cytomegalovirus (HCMV) (Towne strain)–infected autologous monocytes (donor H-22) stimulate both IE1- and pp65-specific cytotoxic T lymphocyte (CTL).

Effectors restimulated with	Lysis (%) of autologous B-LCL targets infected with[a]				Lysis (%) of mismatched B-LCL targets infected with		
	Vac-pp65	Vac-IE1	Vac-IE1 + Vac-pp65	Wt-Vac	Vac-pp65	Vac-IE1	Vac-IE1 + Vac-pp65
Canarypox-pp65–infected PBMC	39 ± 3.2	NT	58 ± 3.9	3 ± 1.3	5 ± 1.6	NT	9.8 ± 2.1
Canarypox-IE1–infected PBMC	NT	65 ± 2.8	68 ± 4.5	6 ± 1.5	NT	4.3 ± .8	10.9 ± 2.6
HCMV-infected monocytes	40 ± 2.8	45 ± 2.4	65.9 ± 6.5	17 ± 2.3	18 ± 1.6	11 ± 1.2	21 ± 2.5

NOTE. Percentage lysis (±SD) at effector-to-target ratios of 50 : 1. B-LCL, B-lymphoblastoid cell line; PBMC, peripheral blood mononuclear cells; NT, not tested.

[a] Target cells were coinfected with Vac-pp65 and Vac-IE1 or with Vac-pp65 combined with WT-Vac or Vac-IE1 combined with WT-Vac or with WT-Vac only (final multiplicity of infection, 10).

cated 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 (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).

Phenotype of effector cells. The phenotypic characteristics of the pp65-, IE1-exon4–, pp150-, and gB-specific effector cells were examined after 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 (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; figure 5*A*). 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 $CD8^+$, whereas the $CD4^+$ population showed no significant pp65 CTL activity (figure 5*B*). 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), because killing of specific target cells after depletion of $CD4^+$ cells was similar to the killing of non-depleted cells (table 4). gB-specific effector cells, however, lost their activity after $CD4^+$ depletion, but not after $CD8^+$ depletion (donor H-35; table 4). The $CD8^+$ phenotype of IE1-exon4 and pp150 CTLs and the $CD4^+$ phenotype of gB-specific CTLs are consistent with the finding that IE1-exon4 and pp150 are presented by class I alleles, whereas gB is presented by class II alleles.

Discussion

In the present study, we analyzed the HCMV-specific CTL responses of naturally seropositive healthy adult blood donors, comprising different racial groups in different geographic areas with a variety of HLA haplotypes, to determine the prevalence of CTLs specific to various HCMV proteins. We used in vitro cytotoxicity assays, 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 some 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. In the experiments described earlier, no obvious differences were observed among racial groups of different geographic areas in the cytotoxic pattern of response to the specific antigens. However, CTL responses did correlate with HCMV NA levels, that is, donors with HCMV NA titers $\geq$1 : 128 were more likely to have CTLs positive for multiple CMV antigens, whereas all but 2 of the moderately seropositive donors (HCMV NA titers $\leq$1 : 96) had CTLs specific for only pp65 or for pp65 and IE1-exon4 (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 [32], and pp65 was identified as the immunodominant CTL target [8, 11]. 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 [32]. In a study to determine pp65-specific CTL precursor frequencies, Wills et al. [12] 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

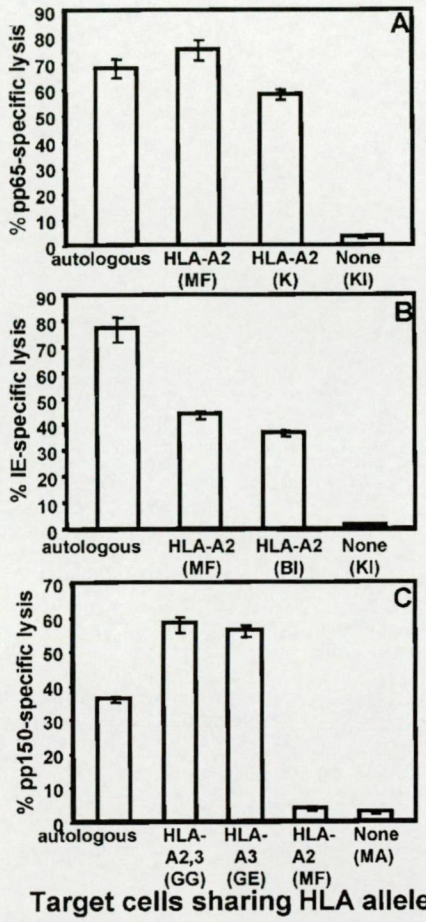

Figure 4. Human lymphocyte antigen (HLA)–class I restriction of pp65- *(A)*, IE1- *(B)*, and pp150- *(C)* specific cytotoxic T lymphocytes (CTLs) of donor H-36. Peripheral blood mononuclear cells (PBMC) were restimulated in vitro, and effector cells were tested against a panel of partially HLA-matched and HLA-mismatched targets expressing individual cytomegalovirus proteins. Percentage specific lysis of target cells is shown at an effector-to-target ratio of 50 : 1. *A,* pp65-Specific effector cells of donor H-36 tested against partially HLA-matched targets of donor MF and donor K sharing HLA-A2 alleles with donor H-36, and HLA-mismatched targets of donor KI. *B,* IE1-specific effector cells of donor H-36 tested against partially HLA-matched targets of donor MF and donor BI sharing HLA-A2 alleles with donor H-36, and HLA-mismatched targets of donor KI. *C,* pp150-Specific effector cells of donor H-36 tested against partially HLA-matched targets of donor GG sharing HLA-A2 and A3 alleles with donor H-36, partially HLA-matched targets of donor GE sharing HLA-A3 alleles, partially HLA-matched targets of donor MF sharing HLA-A2 alleles, and HLA-mismatched targets of donor MA.

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 [11], A2, B7, B8, and B35 [12] were identified as pp65 epitope-presenting alleles. In our panel, by using partially HLA-matched target cells, 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 our use of EBV-transformed B lymphoblastoid cells that express both MHC class I and II antigens, in contrast to the use by other investigators of mainly fibroblast target cells that express only MHC class I alleles, which do not enable detection of $CD4^+$ effector cells. We also found donor variability in the phenotype of pp65-specific CTLs, because donors H-36 and H-33 showed only $CD8^+$ effector cells. The percentage of pp65-specific CTL responders with both $CD4^+$ and $CD8^+$ effector cells is unclear. 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 expressed 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 peptide has been mapped to IE1 [33–36]. In 1 study, a high proportion of HCMV-specific CTLs recognized IE1 in 2 donors tested [15]. IE1-specific CTLs were also shown in bulk culture assays [8, 19, 32], but only in a few donors, 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 [12]. 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 [20]. In our study, IE1-exon4–specific CTLs were nearly as prevalent as pp65-specific CTLs, with similar precursor frequencies. Moreover, this response was maintained over a prolonged period of

Table 3. Human lymphocyte antigen (HLA) alleles presenting human cytomegalovirus (HCMV) pp65, IE1-exon4, and pp150.

	HLA class I alleles presenting HCMV antigens		
Donor	pp65	IE1-exon4	pp150
H-22	B7	B8	
H-33		B70	
H-35	A68	B7	
H-36	A2	A2	A3
BJ	A1, A2	A1	
TK	B12/44	A3	B14
KI	B7	B7, B18	
DF	A28		
CsK		B18	
NJ		B18	
BI	A2		

NOTE. Effector cells were tested against a panel of partially HLA-matched and HLA-mismatched targets expressing cytomegalovirus proteins.

time, as shown in 10 donors, specifically for donor H-35 who showed IE1-exon4– and pp65-specific lysis of target cells at multiple time points during a 27-month period. This result suggests that detectability of the IE1-exon4–specific CTLs is not a random phenomenon due to a HCMV-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. Note that 2 donors in our highly seropositive study group had IE1-exon4–specific CTLs but not pp65-, pp150-, or gB-specific effector cells. It is possible that restimulation of individual HCMV-specific CTLs by use of canarypox recombinants enhances the detection of IE1-exon4–specific CTLs, compared with the use of vaccinia-IE1 recombinants or CMV-infected fibroblasts for restimulation. Gilbert et al. [18] 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 kinase activity. In our study, IE1-specific effector cells readily lysed target cells coinfected with Vac-pp65 and Vac-IE1. 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 that 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 [20], inclusion of the full-length IE1 protein in an HCMV vaccine may be problematic because of the known transactivating ability of the HCMV-IE1 proteins [37]. 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 (authors' unpublished observations). Recent data show that IE1-exons2 and 3, but not exon4, are required for binding to p107, a member of a family of cell-

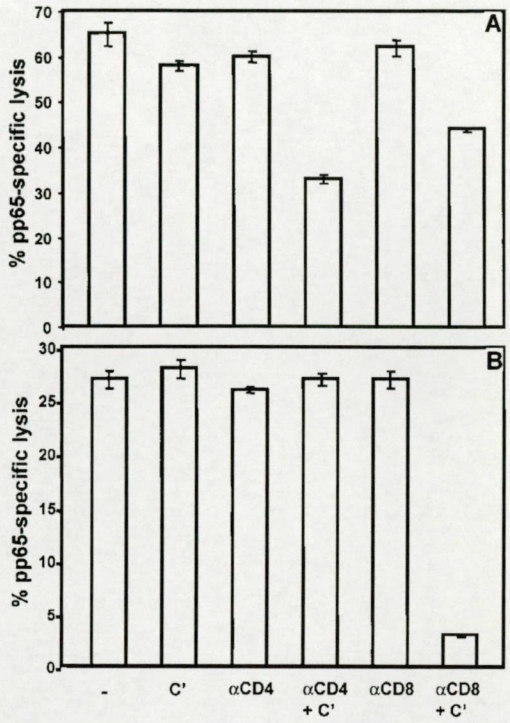

Figure 5. Phenotype of pp65 cytotoxic T lymphocyte (CTL) of donors H-35 *(A)* and H-36 *(B)*. Bulk CTL cultures were established from fresh peripheral blood mononuclear cells. After 11- to 13-day culture in vitro, effector cells were harvested, and aliquots of 3×10^6 cells were depleted of $CD4^+$ lymphocytes by anti-CD4 monoclonal antibody (MAb) + C′, or of $CD8^+$ lymphocytes by anti-CD8 MAb + C′. Percentage specific lysis is shown at an effector-to-target ratio of 30 : 1.

Table 4. Phenotype of pp65-, IE-, pp150-, and gB-specific cytotoxic T lymphocyte (CTL) of selected donors.

Donor	CTL specificity	Specific lysis (%) of target cells by effector cells treated with[a]					
		—	C′	Anti-CD4	Anti-CD4 + C′	Anti-CD8	Anti-CD8 + C′
H-35	IE1 exon4**	38 ± 2.3	41 ± 2.6	46 ± 3.1	47 ± 3.4	36 ± 2.9	10 ± 1.9
H-36	IE1 exon4*	18 ± 1.5	ND	17 ± 1.1	19 ± 2.3	21 ± 2.6	1 ± .8
H-36	pp150**	33 ± 3.2	35 ± 2.5	39 ± 1.9	54 ± 4.3	37 ± 2.1	11 ± 1.6
H-33	pp65**	52 ± 6.6	ND	ND	60 ± 5.4	ND	13 ± 1.8
H-35	gB**	18 ± 2.4	22 ± 1.8	18 ± 1.3	2 ± .8	20 ± 2.2	25 ± 1.9

NOTE. Human cytomegalovirus (HCMV) antigen-specific lysis (±SD) is shown at effector-to-target ratios (E : T) of 20 : 1 (*) and of 30 : 1 (**). ND, not done.

[a] Peripheral blood mononuclear cells (PBMC) were restimulated in vitro with ALVAC-recombinant–infected autologous PBMC, after 8–12 day in vitro culture effector cells were depleted of $CD4^+$ and $CD8^+$ lymphocytes, and their IE1-exon4–, pp65-, pp150-, or gB-specific CTL activity tested as described in Materials and Methods.

cycle inhibitors [38], and that deletion of exon3 eliminates transactivation by IE1 [39]. Canarypox recombinants that express only exon4 or most of exon3 and exon4 were equivalent in their ability to stimulate IE1-specific CTLs from several HCMV seropositive donors (authors' unpublished observations). Taken together, these observations suggest that expression of 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 [15, 40]. Hopkins et al. [19] showed gB-specific CTLs in 4 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 [8, 32]. In donor H-35, showing 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. Together, the evidence indicates that gB-specific CTLs characterized as $CD8^+$ or $CD4^+$ cells are induced by natural HCMV infection and can be shown in in vitro CTL assays. Thus, gB protein can serve as an important inducer of NAs in the majority of the population and 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 [8, 11, 13]. Our studies indicated that pp150 was less dominant than pp65 or IE1-exon4 protein, that is, pp150-specific CTLs were detected in only 30% of seropositive donors, with A3 and B14 alleles presenting pp150 to $CD8^+$ cells.

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.

Our study shows that (1) regardless of racial and geographical distribution of the population, IE1-exon4 protein is nearly as prevalent a CTL target as is pp65; (2) gB- and pp150-specific CTLs are detectable in about one-third of the seropositive donors; and (3) pp28 is not an immunodominant CTL target. These results, as well as our findings on the phenotype of HCMV protein-specific CTLs and the presenting alleles, 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.

Acknowledgments

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References

1. Hillyer CD, Snydman DR, Berkman EM. The risk of cytomegalovirus infection in solid organ and bone marrow transplant recipients: transfusion of blood products. Transfusion 1990;30:659–66.
2. Zaia JA. Epidemiology and pathogenesis of cytomegalovirus disease. Semin Hematol 1990;27:5–10.
3. Stagno S, Reynolds DW, Huang ES, Thames SD, Smith RJ, Alford CA. Congenital cytomegalovirus infection. N Engl J Med 1977;296:1254–8.
4. Quinnan GV, Kirmani N, Rook AH, et al. Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non–T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus in bone marrow transplant recipients. N Engl J Med 1982;307:7–13.
5. Quinnan GV, Burns WH, Kirmani N, et al. HLA restricted cytotoxic T lymphocytes are an early immune response and important defense mechanism in cytomegalovirus infections. Rev Infect Dis 1984;6:156–63.
6. Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood 1991;78:1373–80.
7. 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.
8. Riddell SR, Gilbert MJ, Li C-R, Walter BA, Greenberg PD. Reconstitution of protective $CD8^+$ cytotoxic T lymphocyte responses to human cytomegalovirus in immunodeficient humans by the adoptive transfer of T cell clones. In: Michelson S, Plotkin SA, eds. Multidisciplinary approach to understanding cytomegalovirus disease. Amsterdam: Elsevier Science Publishers, 1993: 155–64.
9. Walter EA, Philip MD, Greenberg PD, 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.
10. Gilbert MJ, Riddell SR, Li C-R, Greenberg PD. Selective interference with class I major histocompatibility complex presentation of the major immediate-early protein following infection with human cytomegalovirus. J Virol 1993;67:3461–9.
11. McLaughlin-Taylor E, Pande H, Forman S, et al. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for $CD8^+$ virus-specific cytotoxic T lymphocytes. J Med Virol 1994;43: 103–10.
12. Wills MR, Carmichael AJ, Mynard K, et al. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J Virol 1996;70:7569–79.
13. Boppana SB, Britt WJ. Recognition of human cytomegalovirus gene products by HCMV-specific cytotoxic T cells. Virology 1996;222:293–6.
14. Diamond DJ, York J, Sun J-Y, Wright CL, Forman SJ. Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. Blood 1997;90:1751–67.
15. Borysiewicz LK, Hickling JK, Graham S, et al. Human cytomegalovirus-specific cytotoxic T cells. J Exp Med 1988;168:919–31.
16. Alp NJ, Allport TD, Van-Zanten J, Rodgers B, Sissons JGP, Borysiewicz

LK. Fine specificity of cellular immune responses in humans to human cytomegalovirus immediate-early 1 protein. J Virol 1991;65:4812–20.
17. Utz U, Koenig S, Coligan JE, Biddison WE. Presentation of three different viral peptides, HTLV-1 tax, HCMV gB, and influenza virus M1, is determined by common structural features of the HLA-A2.1 molecule. J Immunol 1992;149:214–21.
18. Gilbert MJ, Riddell SR, Plachter B, Greenberg PD. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. Nature 1996;383:720–2.
19. Hopkins JI, Fiander AN, Evans AS, Delchambre M, Gheysen D, Borysiewicz LK. Cytotoxic T cell immunity to human cytomegalovirus glycoprotein B. J Med Virol 1996;49:124–31.
20. Kern F, Surel IP, Faulhaber N, et al. Target structures of the CD8$^+$-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. J Virol 1999;73:8179–84.
21. Landini MP, Michelson S. Human cytomegalovirus proteins. Prog Med Virol 1988;35:152–85.
22. Gönczöl E, Furlini G, Ianacone J, Plotkin SA. A rapid microneutralization assay for cytomegalovirus. J Virol Methods 1986;14:37–41.
23. Endresz V, Kari L, Berencsi K, et al. 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;17:50–8.
24. Akrigg A, Wilkinson GWG, Oram JD. The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Res 1985;2: 107–21.
25. Gönczöl E, Berencsi K, Pincus S, et al. Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate. Vaccine 1995;13:1080–5.
26. Panicali D, Davis SW, Mercer SR, Paoletti E. Two major DNA variants present in serially propagated stocks of the WR strain of vaccinia virus. J Virol 1981;37:1000–10.
27. Perkus ME, Goebel SJ, Davis SW, et al. Vaccinia virus host range genes. Virology 1990;179:276–86.
28. Lefkovits I, Waldmann H. Limiting dilution analysis of cells of the immune system. Cambridge: Cambridge University Press, 1979:60–82.
29. Rice GPA, Schrier RD, Oldstone MBA. Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. Proc Natl Acad Sci USA 1984;81:6134–8.
30. Fish KN, Britt W, Nelson JA. A novel mechanism for persistence of human cytomegalovirus in macrophages. J Virol 1996;70:1855–62.
31. Lathey JL, Spector SA. Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. J Virol 1991;65:6371–5.
32. Riddell SR, Rabin M, Geballe AP, Britt WJ, Greenberg PD. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. J Immunol 1991;146:2795–804.
33. Reddehase MJ, Koszinowski UH. Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature 1984;312:369–71.
34. Reddehase MJ, Mutter W, Munch K, Buring H-J, Kosinowski UH. CD8$^+$ T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J Virol 1987;61:3102.
35. Reddehase MJ, Rothbard JB, Koszinowski UH. A pentapeptide as minimal antigenic determinant for MHC class-I–restricted T lymphocytes. Nature 1989;337:651–3.
36. Holtappels R, Podlech J, Geginat G, Steffens H-P, Thomas D, Reddehase MJ. Control of murine cytomegalovirus in the lungs: relative but not absolute immunodominance of the immediate-early 1 nonapeptide during the antiviral cytolytic T-lymphocyte response in pulmonary infiltrates. J Virol 1998;72:7201–12.
37. Kim S, Yu SS, Lee IS, et al. Human cytomegalovirus IE1 protein activates AP-1 through a cellular protein kinase(s). J Gen Virol 1999;80:961–9.
38. Johnson RA, Yurochko AD, Poma EE, Zhu L, Huang E-S. Domain mapping of the human cytomegalovirus IE1-72 and cellular p107 protein-protein interaction and the possible functional consequences. J Gen Virol 1999;80 :1293–1303.
39. Pajovic S, Wong EL, Black AR, Azizkhan JC. Identification of a viral kinase that phosphorylates specific E2Fs and pocket proteins. Mol Cell Biol 1997;17:6459–64.
40. Liu Y-NC, Klaus A, Kari B, Stinski MF, Eckhardt J, Gehrz RC. The N-terminal 513 amino acids of the envelope glycoprotein gB of human cytomegalovirus stimulates both B- and T-cell immune responses in humans. J Virol 1991;65:1644–8.

II.

CONCISE COMMUNICATIONS

A Canarypox Vector Expressing Cytomegalovirus (CMV) Glycoprotein B Primes for Antibody Responses to a Live Attenuated CMV Vaccine (Towne)

Stuart P. Adler,[1] Stanley A. Plotkin,[2] Eva Gonczol,[3,4] Michel Cadoz,[5] Claude Meric,[5] Jian Ben Wang,[1] Pierre Dellamonica,[6] Al M. Best,[1] John Zahradnik,[2] Steve Pincus,[7] Klara Berencsi,[3] William I. Cox,[7] and Zsofia Gyulai[3]

[1]*Medical College of Virginia/Virginia Commonwealth University, Richmond;* [2]*Pasteur Mérieux Connaught USA, Swiftwater, and* [3]*The Wistar Institute, Philadelphia, Pennsylvania;* [4]*Albert Szent-Gyorgyi Unversity, Szeged, Hungary;* [5]*Pasteur Mérieux Connaught France, Marcy l'Étoile, and* [6]*Hôpital de l'Archet, Nice, France;* [7]*Virogenetics, Troy, New York*

To develop a vaccine against cytomegalovirus (CMV), a canarypox virus (ALVAC) expressing CMV glycoprotein (gB) was evaluated alone or in combination with a live, attenuated CMV vaccine (Towne). Three doses of $10^{6.5}$ $TCID_{50}$ of ALVAC-CMV(gB) induced very low neutralizing or ELISA antibodies in most seronegative adults. However, to determine whether ALVAC-CMV(gB) could prime for antibody responses, 20 seronegative adults randomly received either $10^{6.8}$ $TCID_{50}$ of ALVAC-CMV(gB) or $10^{6.8}$ $TCID_{50}$ of ALVAC-RG, expressing the rabies glycoprotein, administered at 0 and 1 month, with all subjects receiving a dose of $10^{3.5}$ pfu of the Towne vaccine at 90 days. For subjects primed with ALVAC-CMV(gB), neutralizing titers and ELISA antibodies to CMV(gB) developed sooner, were much higher, and persisted longer than for subjects primed with ALVAC-RG. All vaccines were well tolerated. These results demonstrate that ALVAC-CMV(gB) primes the immune system and suggest a combined-vaccine strategy to induce potentially protective levels of neutralizing antibodies.

A live, attenuated cytomegalovirus (CMV) vaccine (Towne) has been evaluated in normal volunteers and in renal transplant patients [1, 2]. Towne was immunogenic, reduced the severity and incidence of CMV-associated disease among seronegative recipients of seropositive kidneys [2], and did not reactivate in these patients [3]. Although Towne protected against low doses of an unattenuated strain of CMV in an artificial challenge study, at a reduced dose that induced low neutralizing titers, Towne failed to prevent child-to-mother transmission of CMV, whereas naturally seropositive women were protected [4, 5].

Avipox viruses, such as canarypox (ALVAC), are candidate vaccine vectors because the ALVAC genome accepts large amounts of foreign DNA and will direct the synthesis of multiple foreign proteins. ALVAC does not produce progeny in mammalian cells or nonavian species. It does, however, elicit protective immune responses in nonavian species [6, 7]. Moreover, live ALVAC does not produce disease in healthy subjects or immunosuppressed patients [8].

To develop a vaccine strategy for obtaining high levels of neutralizing antibodies against CMV in humans, we evaluated the safety and immunogenicity of an ALVAC recombinant expressing the major CMV envelope glycoprotein (gB) given alone or in combination with Towne vaccine.

Materials and Methods

Trial A. The first study, conducted at the Hôpital de l'Archet (Nice, France), enrolled 20 healthy male and female volunteers (10 seronegative and 10 seropositive), 18–50 years old. By use of an open-label format, each volunteer received three doses of ALVAC-CMV(gB) vaccine (batch S2723), each dose containing $10^{6.5}$ $TCID_{50}$ of canarypox. The second and third doses were administered 28 and 180 days after the initial dose. Volunteers were monitored for 30 min after each injection and then were examined by a study physician on days 1, 2, 3, and 7 after each injection, to monitor local and systemic reactions. Blood samples were taken on days −14, 0, 2, 7, 28, 30, 35, 56, 180, 182, 187, and 208. Samples of saliva and urine were taken on days −14, 0, 7, 28, 35, 56, 180, 187, and 208.

Trial B. The second vaccine study, conducted in Richmond, VA, enrolled 20 healthy CMV-seronegative male and female volunteers, 20–43 years old. By use of a double-blind format, volunteers were randomized to receive either an initial injection of $10^{6.8}$ $TCID_{50}$ ALVAC-CMV(gB) (batch S3145) and a second, similar injection 1 month later or a first injection of $10^{6.8}$ $TCID_{50}$ of AL-

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Informed consent was obtained from all subjects. The human experimentation guidelines of the US Department of Health and Human Services and those of Medical College of Virginia/Virginia Commonwealth University were followed in the conduct of the clinical research. The protocol was approved by the Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédical (CCPPRB) of Nice, France.

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Reprints and correspondence: Dr. Stuart P. Adler, Medical College of Virginia, P.O. Box 980163, Richmond, VA 23298-0163 (SADLER@HSC.VCU.EDU).

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VAC-RG (batch S3106) and a second, similar injection 1 month later. Both groups received a single 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 Richmond 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 interval history was obtained.

Vaccines. The Towne vaccine (lot C-107) was prepared by Program Resources (Rockville, MD). The seed strain was Towne pool, passage 131. The vaccine pool was prepared in cultures of MRC-5 cells. A harvest pool was clarified by centrifugation at 1200 *g* for 20 min and then aliquoted into 3 mL vials for lyophilization. The vials were stored at 2°C–8°C for 19 days, then at −20°C, and finally at −70°C.

The mean infectivity titer was $10^{3.5}$ pfu/lyophilized vial (0.5 mL), which constituted a single dose. Before use, the vaccine was reconstituted in sterile water, maintained on ice, and administered subcutaneously in the deltoid region within 1 h of reconstitution.

ALVAC was developed by Virogenetics (Troy, NY) and manufactured by Pasteur Mérieux Connaught (Marcy l'Étoile, France). ALVAC-CMV(gB), laboratory designation vCP139, is a recombinant canarypox virus encoding the full-length gB gene of the Towne strain of CMV, whereas ALVAC-RG, laboratory designation vCP65, expresses the glycoprotein G gene of the ERA strain rabies virus. Both vaccines were prepared in primary chick embryo fibroblasts derived from specific pathogen-free eggs. Clarified lysates of infected cells were diluted in serum-free virus stabilizer and lyophilized. Titrations were performed on QT35 cells by the endpoint dilution method. Batches S3106 (ALVAC-RG), S2723 (ALVAC-CMV[gB]), and S3145 (ALVAC-CMV[gB]), with titers of $10^{6.8}$, $10^{6.5}$, and $10^{6.8}$ $TCID_{50}$ per dose, respectively, were used in the trials. Both recombinants were tested in small animals [9], and ALVAC-RG was previously tested in humans and has an extended record of safety [8]. The lyophilized preparations were reconstituted in 1.0 mL of sterile water. The vaccines were given by injection (1.0 mL) into the deltoid muscle within 15 min of reconstitution.

Laboratory methods. Seronegativity prior to enrollment was established by measuring IgG to CMV by use of an enzyme immunoassay, as described elsewhere [10]. Neutralizing antibodies were determined by a standard reduction assay [11]. A titer was defined as the reciprocal of the highest dilution of serum that fully inhibited viral cytopathic effect (CPE), compared with 100% CPE in control wells.

IgG antibodies to gB in sera were measured by an enzyme immunoassay, as described elsewhere [12]. Antibodies to the canarypox vector (ALVAC) were measured at Pasteur Mérieux Connaught by use of an enzyme immunoassay with plates coated with purified ALVAC-CPpp virus (ALVAC vector without insert).

CMV-specific lymphocyte responses were determined as described elsewhere [13]. Lymphocyte proliferation responses were expressed as stimulation indices, defined as the ratio of counts per minute (CPM) in CMV antigen–stimulated cultures to CPM in control antigen–stimulated cultures. To measure lymphocyte responses to CMV(gB), purified gB was used at 3.0 μg/mL.

Urine and saliva samples of trial B 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 CMV.

Statistical analysis. 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).

Results

Safety and reactogenicity. No severe adverse reactions occurred in either trial. Local and systemic reactions were mild, consisting mainly of pain and redness at the injection site. All three immunizations were well tolerated in all recipients. Seropositive and seronegative subjects had the same frequency of local and systemic reactions.

In trial B, 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.

Immunogenicity. In trial A, three doses of ALVAC-CMV(gB) administered on days 0, 28, and 180 failed to increase neutralizing titers among the 10 seropositive subjects or to induce significant neutralizing titers among the 10 seronegative subjects. No intercurrent CMV infections occurred.

We performed a second trial to determine whether ALVAC-CMV(gB), while not eliciting detectable levels of antibodies itself, could prime an immune response to gB. Two groups 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.

After two injections of ALVAC and before booster with Towne on day 90, a low increase ($P < .057$) 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 CMV-specific neutralizing antibodies was observed in ALVAC-RG recipients (table 1).

After administration of the Towne vaccine on day 90, the ALVAC-CMV(gB) group developed significantly higher mean ELISA titers against CMV gB [$F(9144) = 4.03$, $P < .0001$] and mean neutralizing titers [$F(9142) = 8.9$, $P < .0001$] 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

Table 1. ELISA antibody responses to cytomegalovirus gB and neutralizing titers in trial B.

Days after first dose[a]	IgG to gB (reciprocal titers) ALVAC-gB (n=10) mean (range)	IgG to gB (reciprocal titers) ALVAC-RG (n=8) mean (range)	IgG to gB (reciprocal titers) Ratio[b]	Neutralizing titers (reciprocal titers) ALVAC-gB (n=10) mean (range)	Neutralizing titers (reciprocal titers) ALVAC-RG (n=8) mean (range)	Neutralizing titers (reciprocal titers) Ratio[b]
0	115 (100–400)	119 (100–400)	1.0	1.0 (1–1)	1.0 (1–1)	1.0
30	163 (100–1600)	100 (100–100)	1.6	1.1 (1–3)	1.0(1–1)	1.1
60	303 (100–1600)[c]	119 (100–400)	2.5	1.6 (1–10)	1.0(1–1)	1.6
90	303 (100–1600)[c]	100 (100–100)[d]	3.0	1.2 (1–8)	1.0 (1–1)	1.2
97	566 (100–1600)	141 (100–1600)[d]	4.0	1.5 (1–8)	1.0 (1–1)	1.5
104	3676 (100–6400)	141 (100–1600)[d]	26.0	10 (1–25)	1.1 (1–2)[d]	9.1
120	38,802 (100–409,600)	1345 (100–6400)[d]	28.8	207 (39–670)	23 (15–39)[d]	9.1
180	89,144 (25,600–409,600)	15,222 (100–102,400)[d]	5.9	266 (105–839)	89 (56–128)[d]	3.0
270	58,813 (25,600–409,600)	10,764 (100–102,400)[d]	5.5	109 (46–419)	36 (2–128)[d]	3.0
360	22,286 (6400–102,400)	4526 (100–25,600)[d]	4.9	96 (26–364)	33 (2–257)[d]	2.9

NOTE. ALVAC, canarypox virus; gB, glycoprotein.

[a] The ALVAC vaccines were given at days 0 and 30 and Towne at day 90.

[b] At each timepoint, ratios are the values of the ALVAC-gB group divided by the ALVAC-RG group.

[c] A value of 1 was used in the calculation of the geometric means for neutralizing titers <1 : 4 (or in some cases <1 : 8) and gB titers <1 : 100.

[d] Significantly lower ($P < .05$) than the values for the ALVAC-gB group at the corresponding time.

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 (table 2). An IgG antibody response to the canarypox vector was induced in all vaccinees in both trials (data not shown).

Priming with ALVAC-CMV(gB) allowed the Towne vaccine to induce peak ELISA titers to gB and CMV neutralizing titers at levels significantly higher than those observed among the naturally seropositive subjects in trial A. For the naturally seropositive subjects at entry (day 0), 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) in trial B ($P = .03$). The same was true for neutralizing titers. At entry, the naturally seropositive subjects in trial A 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) in trial B ($P = .008$).

Lymphoproliferative responses. Lymphoproliferative responses were measured monthly in trial B over the first 180 days in each subject by use of two antigens: purified gB and extracts of Towne CMV–infected cells. There were no statistically significant differences in the mean lymphoproliferative responses between the group primed with ALVAC-CMV(gB) and the group primed with ALVAC-RG (table 2).

Discussion

The results of these trials demonstrated that an ALVAC recombinant containing the gene coding for CMV gB could prime the immune system to produce a strong neutralizing response to the gB produced by an attenuated CMV virus.

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 CMV Towne vaccine.

The very low levels of gB-specific IgG and neutralizing activities induced by ALVAC-CMV(gB) in humans were unexpected, because this vaccine induces detectable levels of these activities in mice and guinea pigs [9]. 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 CMV gB is lower in humans than in small mammals, CMV gB may be a poorer immunogen in humans than in laboratory animals. The observation that two immunizations of ALVAC-CMV(gB) induced a low immune response in trial B but none in trial A can probably be explained by a difference in the titer of the two batches of vaccine used.

An important observation made in this trial is that this ALVAC vaccine could prime for an immune response to a live, attenuated vaccine (Towne) and induced peak ELISA anti-gB

Table 2. Lymphoproliferative responses to either cytomegalovirus gB antigen or Towne antigen in trial B.

Days after first dose	Mean stimulation index (range) Towne antigen ALVAC-gB (n=10)	Towne antigen ALVAC-RG (n=8)	gB antigen ALVAC-gB (n=10)	gB antigen ALVAC-RG (n=8)
0	1.9 (1–5.6)	1.6 (1–3.5)	1.3 (1–3.2)	1.3 (1–1.9)
30	2.7 (1–9.3)	2.3 (1.4–3.5)	1.6 (1–6.7)	1.5 (1–3.4)
60	2.1 (1–6.2)	2.2 (1.6–4.1)	1.1 (1–1.7)	1.5 (1–6.4)
90	3.2 (1.3–12)	3.1 (1.4–9)	2.6 (1–8.6)	1.6 (1–4.1)
120	9.7 (1.8–113.7)	9.6 (1.7–122)	2.3 (1–46.2)	3.4 (1.2–8.6)
180	19.1 (3.7–74.3)	21.0 (3.9–66.2)	6.6 (1.5–53.1)[a]	3.9 (1–22.9)

NOTE. ALVAC, canarypox virus; gB, glycoprotein.

[a] Not significantly higher ($P > .05$) than for those primed with ALVAC-RG.

titers and neutralizing titers at levels equal to or higher than those observed among the naturally seropositive subjects.

For CMV, the majority of the immunogenic neutralizing epitopes occur on the gB envelope glycoprotein of the viral particle [14], and the data of the current study are consistent with this, as both antibodies to CMV gB and neutralizing activity were boosted to high levels after priming with CMV gB.

Lymphoproliferative responses to the Towne vaccine were not primed by gB. We previously found that induction of maximal lymphoproliferative responses to Towne antigen required less antigen than that required to induce maximal antibodies, suggesting that the Towne vaccine is an effective inducer of lymphoproliferative responses [5]. The lack of priming of lymphoproliferative responses by ALVAC-CMV(gB) suggests that gB expressed in ALVAC-infected cells is an inefficient inducer of lymphoproliferative responses.

Results of this trial confirm similar trials using ALVAC expressing HIV-1 MN gp160 [15]: ALVAC alone induces a weak antibody response but primes for subsequent exposure to HIV envelope.

We previously evaluated the ability of the Towne strain of CMV to prevent the child-to-mother transmission of CMV [5]. Women who were naturally seropositive appeared to be protected from acquiring a CMV 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, we observed that naturally seropositive adults and those with vaccine-induced immunity who had serum neutralizing titers $\geq 1:64$ also had detectable levels of IgG antibodies to CMV gB in nasal washes and saliva [12]. Because gB contains the majority of neutralizing epitopes, we proposed that serum neutralizing titers $>1:64$ would be necessary for protection against wild-type infection [12]. 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 $>1:64$. These results define a vaccine strategy to induce potentially protective levels of neutralizing antibodies. The recall antigen could be either a live virus or, potentially, a subunit antigen.

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References

1. Plotkin SA, Starr SE, Friedman HM, et al. Effect of Towne live virus vaccine on cytomegalovirus disease after renal transplant. Ann Intern Med 1991;114:525–31.
2. Balfour HH Jr, Welo PK, Sachs GW. Cytomegalovirus vaccine trial in 400 renal transplant candidates. Trans Proc 1985;17:81–3.
3. Plotkin SA, Huang E-S. Cytomegalovirus vaccine virus (Towne strain) does not induce latency. J Infect Dis 1985;152:395–7.
4. Plotkin SA, Starr SE, Friedman HM, Gönczöl E, Weibel RE. Protective effects of Towne cytomegalovirus vaccine against low-passage cytomegalovirus administered as a challenge. J Infect Dis 1989;159:860–5.
5. Adler SP, Starr SE, Plotkin SA, et al. Immunity induced by a primary cytomegalovirus infection protects against secondary infection among women of childbearing age. J Infect Dis 1995;171:26–32.
6. Baxby D, Paoletti E. Potential use of nonreplicating vectors as recombinant vaccines. Vaccine 1992;10:8–9.
7. Taylor J, Trimarchi C, Weinberg R, et al. Efficacy studies on a canarypox-rabies recombinant virus. Vaccine 1991;9:190–3.
8. Plotkin SA, Cadoz M, Meignier B, et al. The safety and use of canarypox vectored vaccines. Dev Biol Stand 1995;84:165–70.
9. Gönczöl E, Berencsi K, Pincus S, et al. Preclinical evaluation of an ALVAC (canarypox)—human cytomegalovirus glycoprotein B vaccine candidate. Vaccine 1995;13:1080–5.
10. Adler SP, McVoy M. Detection of cytomegalovirus antibody by enzyme immunoassay and lack of evidence for and effect resulting from strain heterogeneity. J Clin Microbiol 1986;24:870–2.
11. Gönczöl E, Furlini G, Ianacone J, Plotkin SA. A rapid micro neutralization assay for cytomegalovirus. J Virol Methods 1986;14:37–41.
12. Wang JB, Adler SP, Hempfling S, et al. Mucosal antibodies to CMV glycoprotein B occur following both natural infection and immunization with human cytomegaloviral vaccines. J Infect Dis 1996;174:397–2.
13. Starr SE, Dalton B, Garrabrant T, Paucker K, Plotkin SA. Lymphocyte blastogenesis and interferon production in adult human leukocyte cultures stimulated with CMV antigens. Infect Immun 1980;30:17–22.
14. Britt WJ, Vugler L, Butfiloski EJ, et al. Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response. J Virol 1990;64:1079–85.
15. Clements-Mann ML, Weinhold K, Matthews TJ, et al. Immune responses to human immunodeficiency virus (HIV) type 1 induced by canarypox expressing HIV-1MN gp120, HIV-1SF2 recombinant gp120, or both vaccines in seronegative adults. J Infect Dis 1998;177:1230–46.

III.

A Canarypox Vector–Expressing Cytomegalovirus (CMV) Phosphoprotein 65 Induces Long-Lasting Cytotoxic T Cell Responses in Human CMV-Seronegative Subjects

Klara Berencsi,[1] Zsofia Gyulai,[1] Eva Gönczöl,[1] Steve Pincus,[4,a] William I. Cox,[4] Susan Michelson,[5] Laszlo Kari,[1] Claude Meric,[6] Michel Cadoz,[6] John Zahradnik,[3] Stuart Starr,[2] and Stanley Plotkin[3]

[1]*Wistar Institute and* [2]*Children's Hospital of Philadelphia, Philadelphia, and* [3]*Aventis Pasteur, Swiftwater, Pennsylvania;* [4]*Virogenetics, Troy, New York;* [5]*Institut Pasteur, Paris, and* [6]*Aventis Pasteur, Lyon, France*

The major matrix phosphoprotein 65 (pp65) of cytomegalovirus (CMV) is an important target of HLA-restricted cytotoxic T cells (CTL) after natural infection. A canarypox–CMV pp65 recombinant was studied for its ability to induce CMV pp65–specific CTL, helper T lymphocytes, and antibodies in a phase I clinical trial. Twenty-one CMV-seronegative adult volunteers were randomized to receive immunizations at months 0, 1, 3, and 6 with either canarypox–CMV pp65 or placebo. In canarypox–CMV pp65–immunized subjects, pp65-specific CTL were elicited after only 2 vaccinations and were present at months 12 and 26 in all subjects tested. Cell-depletion studies indicated that the CTL were phenotype $CD8^+$. Peripheral blood mononuclear cells proliferated in response to stimulation with purified pp65, and antibodies specific for pp65 also were detected. Canarypox–CMV pp65 is the first recombinant vaccine to elicit CMV-specific CTL responses, which suggests the potential usefulness of this approach in preventing disease caused by CMV.

Cytomegalovirus (CMV) infection occurs in nearly half the persons in the Western hemisphere and in almost all persons elsewhere. Infection is usually asymptomatic in immunocompetent persons; however, under certain conditions, it may have serious consequences. Each year in the United States, ~8000 CMV-infected infants are born with congenital defects, including deafness, blindness, and mental disorders. Although some data point to the importance of recurrent infection [1], the majority of studies indicate that CMV-induced defects result from a primary maternal CMV infection during pregnancy. In contrast, such defects are rare in children born to mothers who were CMV-seropositive before pregnancy [2, 3], which suggests a protective effect of maternal immunity.

Organ transplantation is also affected by CMV-related complications in 20%–60% of subjects, depending on the immune status of the donor and recipient. A graft from a CMV-seropositive donor may lead to serious and sometimes life-threatening disease in a seronegative recipient; symptoms are usually less severe in a seropositive recipient [4]. The relative significance of cellular and humoral immunity in defense mechanisms against CMV disease is uncertain, but the following observations point to the importance of cellular responses. First, kidney transplant recipients have more frequent and severe CMV infections in the posttransplantation period after the use of antithymocyte globulin or monoclonal antibody (MAb) directed to $CD3^+$ lymphocytes [5, 6]. Second, there is an inverse correlation between the presence of CMV-specific cytotoxic T lymphocytes (CTL) and CMV-related complications of transplant recipients, and CMV-specific T cell immunity after renal transplantation mediates protection from CMV disease by limiting systemic virus load [7–10]. Third, the transfer of T cells obtained from HLA-matched–seropositive donors reduces the incidence of severe CMV disease in bone marrow transplant recipients [11–13].

Infection-induced CTL in naturally infected people target several CMV proteins, including the major matrix protein, phosphoprotein 65 (pp65; UL83), immediate-early 1 (UL123), pp150 (UL32), glycoprotein B (gB; UL55), and glycoprotein H (UL75). pp65 is considered to be the dominant CTL target [14–23] and thus the most likely vaccine candidate to induce CTL-mediated protection against CMV diseases.

Induction of CTL responses by vaccination is best achieved by methods that allow for intracellular processing of antigens such as live attenuated viral vaccines, plasmid DNA carriers, or recombinant viruses. Canarypox recombinants based on an attenuated strain of canarypox virus (ALVAC) [24–26] are well

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Written informed consent was obtained from all subjects. Human experimentation guidelines of the US Department of Health and Human Services and of Children's Hospital, Philadelphia, were followed in the conduct of the clinical research.

Financial support: Aventis Pasteur.

[a] Present affiliation: EluSys Therapeutics, Pine Brook, New Jersey.

Reprints or correspondence: Dr. Stanley Plotkin, Aventis Pasteur, 4650 Wismer Rd., Doylestown, PA 18901 (Stanley.Plotkin@Aventis.com).

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tolerated when administered to humans and induce antibody responses against many viral antigens, including rabies virus glycoprotein [27, 28], measles virus hemagglutinin and fusion proteins [29], and human immunodeficiency virus (HIV) type 1 envelope glycoproteins [30]. CTL responses to HIV glycoproteins were elicited in 30%–39% of vaccinated persons, with 60%–70% positive responses at ≥1 time points [30, 31]. In addition, immunization with an ALVAC vector expressing multiple HIV-1 genes that was followed by a boost with recombinant gp120 resulted in durable CTL responses in all of the encoded genes [32]. We recently reported that canarypox-CMV-gB recombinant primes for antibody responses to the Towne vaccine strain of CMV [33].

The ALVAC vector system offers several advantages, including a genome that can easily accommodate large or multiple foreign genes from heterologous pathogens and the ability to abortively infect mammalian cells. Expression of the foreign gene product is induced in the absence of productive viral replication. Thus, the ALVAC vector system provides a strong safety barrier against potential vaccine-associated complications in humans. In fact, live ALVAC has been administered to immunosuppressed mice and HIV-infected adults without serious adverse effects [25, 26].

The present study was designed to test the ability of a canarypox–CMV pp65 recombinant to elicit pp65-specific CTL and antibody responses in CMV-seronegative humans. We characterized the level and duration of the CTL activity, precursor frequency, lymphoproliferation, and antibody response and determined the phenotype of the cytotoxic effector cells.

Materials and Methods

Study design. This randomized placebo-controlled clinical trial was conducted at Children's Hospital of Philadelphia. Healthy adult volunteers, 18–35 years old, were eligible to participate unless they had ≥1 of the following exclusion criteria: 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 of immunoglobulins, blood or blood products, steroids, oral or parenteral immunosuppressive therapy, or cimetidine within the previous 6 months; prescription medication use (other than oral contraceptives); planned immunization with other vaccines within 7 months of the study period; and pregnancy.

Two months before the first immunization, eligible volunteers were screened for CMV antibodies and for hematologic and biochemical status by means of commercial ELISA kit (Wampole Laboratories). Screenings were done for neutralizing antibodies to CMV [34], as well as for ELISA antibodies to CMV pp65, HIV, hepatitis C, and hepatitis B surface antigen. A complete blood cell count was done, and levels were determined of aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine.

Subjects were admitted to the study if they had no abnormal laboratory results and no detectable CMV antibodies. Inclusion was also proposed to an additional 4 subjects with high titers of CMV neutralizing antibodies. All subjects had 50 mL of blood drawn for preparation of Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines and for HLA typing.

Two months later, the seronegative subjects were randomized to receive canarypox–CMV pp65 (vCP260) or placebo (sterile saccharose reconstituted with diluent) at a ratio of 2:1. The 4 subjects with high titers of CMV neutralizing antibody were immunized with canarypox–CMV pp65 (vCP260). All subjects received 4 immunizations intramuscularly at months 0, 1, 3, and 6. To increase the dose of canarypox–CMV pp65 administered, 2 injections (1 in each arm) were given concomitantly on each occasion. A negative urine pregnancy test was required for female participants before each vaccination. Subjects were seen monthly for the first 7 months after the first immunization and at month 12. During each study visit, blood was drawn for hematologic, biochemical, and immunologic tests. Specific immunologic assays were done on the following schedule: CMV pp65–specific lymphocyte proliferation at months 0, 3, 4, 6, 7, 12, and 26; CTL at months 0, 3, 4, 5, 7, 12, and 26; and ELISA antibodies to CMV pp65 at months 0, 1, 3, 6, and 7.

All subjects were observed for 30 min after each immunization to detect immediate adverse effects. Telephone inquiries were made on days 2 and 9 after each immunization to document reactions. In addition, subjects were required to maintain postvaccination diaries that included a list of systemic reactions (fever, chills, generalized pruritus, urticaria, rash, headache, nausea, vomiting, diarrhea, dizziness, malaise, abdominal pain, arthralgia, myalgia, wheezing, dyspnea, and lymphadenopathy) from days 0 to 7 and local reactions (erythema, induration, bruising, swelling, tenderness, pain, pruritus, and regional adenopathy) at day 3 after each dose. Interim medical histories were obtained at each study visit to detect unreported events, including hospitalizations and medical office or emergency room visits for any reason.

We graded adverse reactions as follows: mild (grade 1), subject is 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; and severe (grade 3), symptom is incapacitating and prevents the subject from working or carrying out usual activities. Statistically significant differences were calculated by using Student's 2-tailed t test. $P < .05$ was considered to be significant.

Viruses. The canarypox–CMV pp65 (ALVAC pp65, vCP260) recombinant containing the pp65 gene derived from the Towne strain of human CMV [23] and the parental canarypox (ALVAC, CPpp) were used for restimulation of CTL effector cells in vitro. We used the WR strain of vaccinia virus (Vac-WR) and Vac-WR–based recombinant encoding CMV pp65 protein (Vac-WR–pp65) to infect target cells in cytotoxicity assays [23]. Vaccinia viruses were propagated and titered on Vero cells (American Type Culture Collection [ATCC]). Immunizations were with batch S3227 of canarypox–CMV pp65, which contained $10^{6.8}$ $TCID_{50}$ per dose. Canarypox viruses were grown on primary chick embryo fibroblast and were titered on QT35 cells.

CTL assays. CTL assays were done at months 0 (26 subjects), 3 (13 subjects), 4 (24 subjects), 5 (13 subjects), 7 (19 subjects), 12 (24 subjects), and 26 (10 subjects). An internal positive control subject (pp65-specific CTL-positive naturally seropositive person) and a negative control subject (CMV-seronegative and pp65-specific CTL negative person) were included in each assay. Control subjects were volunteers from the Wistar Institute's blood donor program; their

pp65-specific CTL reactivity was established in preliminary experiments. Peripheral blood mononuclear cells (PBMC) were typed for major histocompatibility complex class I and class II antigens by means of standard complement-mediated cytotoxicity assays and of polymerase chain reaction testing at the Immunogenetics Laboratory at the Children's Hospital of Philadelphia.

Target cell lines. Autologous B lymphoblastoid cell lines (B-LCLs) were established from each donor by incubation of PBMC with supernatant from the EBV-producing marmoset cell line B95.8 (ATCC). Transformed cell lines were grown in RPMI 1640 with 15% fetal calf serum (FCS; Cansera Atlanta Biologicals) and were cryopreserved until use.

Effector cells. Bulk CTL assays were done, as described elsewhere [23]. In brief, PBMC were separated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals) density gradients. Stimulator cells were prepared by infecting PBMC with canarypox–CMV pp65 or parental canarypox at an MOI of 5. After incubation for 1 h, infected cells were washed and were mixed with responder PBMC at a responder: stimulator ratio of 4:1. The mixture of responder and stimulator cells was placed in 24-well plates at 3.5×10^6 cells in 2 mL/well of culture medium (RPMI 1640; Cellgro; Mediatech), supplemented with 10% FCS (Hyclone [for restimulation cultures] or Cansera Atlanta Biologicals [for chromium release tests]), and 4 m*M* L-glutamine (Sigma Chemical), 60 μg/mL of gentamicin (Sigma), and 5 μ*M* 2-mercaptoethanol (Sigma). PBMC cultures were supplemented with 330 U/mL of interleukin (IL)–7 (R&D Systems) at the start of culture and with 20 U/mL of IL-2 (Genzyme) on days 4 and 8. Cell lines were tested for CTL activity between days 10 and 13 of culture.

Infection and labeling of target cells. We used Vac-WR–pp65 or Vac-WR to infect 2×10^6 to 6×10^6 of B-LCLs at an MOI of 10 for 1 h. Cells were diluted to 10^6 cells/1.5 mL with culture medium, were incubated an additional 16 h, and were washed and labeled with 100 μCi of $Na[^{51}Cr]O_4$ (Amersham Life Sciences) for 1 h. Labeled cells were washed 3 times before use.

Cytotoxicity assays. We measured cytolytic activity of antigen-driven effectors present in bulk cultures established from PBMC in a 4-h ^{51}Cr release assay. Nonadherent effector cells were collected, were washed once, and were added in triplicate to round-bottomed 96-well microtiter plates at effector-to-target ratios (E:T) indicated for individual experiments (typically 50:1, 25:1, 12.5:1, 6:1, 3:1, and 1.5:1). Targets were autologous B-LCLs infected with Vac-WR–pp65 or Vac-WR or heterologous (HLA mismatched) B-LCLs infected with Vac-WR–pp65. Nonspecific background cytolysis was reduced by adding a 30-fold excess of unlabeled, Vac-WR–infected target cells to each well in the assay. Chromium release was measured by gamma counter (Cobra II; Packard Instrument). Percentage of specific lysis was determined as follows: 100 × [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. For each target, spontaneous release was determined from wells containing medium only, and maximal release was calculated from wells containing 1% NP-40. Spontaneous release was always <30%. Effector cells were considered to be positive for CTL activity if percentage of specific lysis of canarypox–CMV pp65–restimulated effector cells on Vac-WR–pp65–infected autologous target cells was ≥10% above background. Background was defined as percentage of specific lysis of canarypox–CMV pp65–restimulated effector cells against Vac-WR–infected autologous target cells or Vac-WR–pp65–infected HLA-mismatched target cells or percentage of specific lysis of ALVAC-restimulated effector cells against Vac-WR–pp65–infected autologous target cells, whichever was highest.

Limiting dilution assay. Fresh or cryopreserved effector PBMC were plated at different cell numbers in 24–30 replicate wells and were cultured with gamma-irradiated (3000 rad delivered by an MKA model 68A irradiator; Shepherd and Associates) autologous PBMC as feeder cells (6×10^4 PBMC/well) and 4×10^3 autologous EBV-transformed lymphoblasts infected with Vac-WR–pp65 for 16 h and gamma irradiated (3000 rad) and UV irradiated (10 min at a distance of 25 cm with a germicidal lamp) as stimulator cells. After 12–14 days, pp65-specific CTL activity was determined for the cells of each well on autologous Vac-WR–pp65–infected, autologous Vac-WR–infected, and HLA-mismatched Vac-WR–pp65–infected target cells. CTL precursor (CTLp) frequency was estimated as the input cell number resulting in 37% negative wells against the pp65-specific target [35]. Linear regression analysis was done, and precursor frequency was determined by solving the equation of the best-fit line, in which the fraction of nonresponding wells equaled 0.37. ^{51}Cr release for each well was considered to be significant at >3 SD above mean lysis observed in control wells (containing only feeder and stimulator cells).

Determination of CTL phenotype. Bulk CTL cultures were established from freshly prepared PBMC. After 10–13 days of culture in vitro, effector cells were harvested, and aliquots of 3×10^6 cells were depleted of $CD4^+$ lymphocytes by anti-CD4 MAb plus complement (C′) or of $CD8^+$ lymphocytes by anti-CD8 MAb plus C′ or of both $CD4^+$ and $CD8^+$ by both antibodies plus C′. Antibodies (Caltag Laboratories) were used at a predetermined concentration (16 μg/mL). Nontoxic rabbit complement (Accurate Chemical) was used at a final dilution of 1:10. Effector cells were incubated with the antibodies at 4°C for 45 min. After a wash with medium, cells were further incubated with complement for 30 min at 37°C and were washed and resuspended in the predepletion volume of medium, to maintain nondepleted cells at the original concentration. CMV antigen-specific lysis was determined in a 4-h ^{51}Cr release assay.

Lymphocyte proliferation assay. Freshly separated PBMC or, in a few cases, cryopreserved PBMC were resuspended to a concentration of 2×10^6 cells/mL in RPMI medium supplemented with 10% autologous plasma (when available) or with human AB serum (Sigma). We added (in triplicate) a 100-μL aliquot containing 2×10^5 cells to wells of a 96-well microtiter U-bottom plate. pp65 purified by high-pressure liquid chromatography from CMV-infected (strain Towne) MRC-5 cells, as described elsewhere [36], or MRC-5 antigen (control antigen) at final concentrations of 2.5, 0.6, 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, 2 identical plates were set up and were incubated for 5 and 6 days. [^{3}H]Thymidine (0.5 μCi) was added to each well for the last 6 h of incubation. Cells were harvested in an automatic cell harvester (Tomtec), and incorporated radioactivity was measured by using a beta plate reader (Packard).

We determined the stimulation index (SI) as the count in the presence of pp65 antigen divided by the count in wells with MRC-5 control antigen. An SI ≥3 and a difference in experimental counts (count in the presence of pp65 antigen minus the count in the

Table 1. Reactogenicity in healthy volunteers after vaccinations with canarypox–cytomegalovirus phosphoprotein 65 (CMV pp65).

Reaction	Reactogenicity in subjects immunized with		
	Canarypox–CMV pp65[a]	Placebo (n = 7)	P[b]
Local			
Any	100	57	.013
Pain	100	29	.001
Tenderness	100	57	.013
Systemic			
Headache	80	43	.049
Myalgia	65	14	.047
Malaise	70	57	.856
Nausea	55	29	.368

NOTE. Data are percentages unless otherwise indicated.

[a] Seronegative subjects (n = 16) and seropositive subjects (n = 4).

[b] $P < .05$ is significant.

presence of control MRC-5 antigen) of 500 were considered to be indicative of a positive response to pp65. A subject's proliferative responses were defined as positive if both criteria were fulfilled with ≥1 concentration of the pp65 antigen after 5 or 6 days of incubation. The highest SI for each time point is given in the Results.

ELISA. pp65- and gB-specific antibodies were determined in a standard ELISA. The gB-specific ELISA plates were coated with 150 ng/well purified gB (Chiron). Serum samples with optical density (OD) values >0.100 at a serum dilution of 1:200 were considered to be positive for gB antibody. In a pp65-specific ELISA, lysates of 293 cells transiently transfected with pΔRC-pp65 were used as coating antigen, and lysates of untransfected 293 cells served as control antigen [37]. The pp65-specific OD values were calculated as follows: OD values obtained in control antigen–coated wells were subtracted from those obtained in pp65 antigen–coated wells. Serum samples with OD values >0.053 (mean OD of serum 200 × dilution + 2 × SD of the originally seronegative subjects at month 0) were considered to be positive for pp65 antibody.

Results

We enrolled 27 volunteers in the trial: 23 were seronegative for CMV and received the vaccine (n = 14) or placebo (n = 9), and 4 were seropositive. Two seronegative canarypox–CMV pp65–immunized subjects did not complete the study: one received an initial dose, and the other received 2 doses. The purpose in immunizing highly seropositive volunteers was primarily to assess the safety of the vaccine in this population. The study subjects (22 women and 5 men) were primarily white (n = 23) and were 21–35 years old.

Reactogenicity. Table 1 shows the rates of the most commonly reported postvaccination adverse reactions for all enrolled subjects, regardless of the number of vaccinations. Elicited reactions recorded on diary cards were more common in subjects who received vaccine than in placebo recipients. There was no evidence that either the severity of the reaction or the frequency of occurrence increased after the administration of successive doses. Reactogenicity did not appear greater in the 4 seropositive subjects than in the seronegative subjects. Mild-to-moderate pain or tenderness lasting 24–48 h were the most frequent local reactions. Headache, malaise, nausea, and myalgia were the most common systemic reactions. All of the vaccinated subjects and

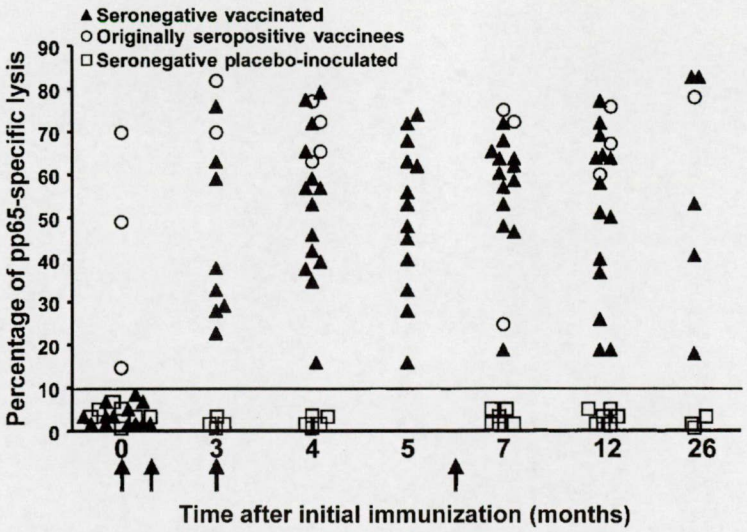

Figure 1. Kinetics of phosphoprotein 65 (pp65)–specific cytotoxic T cell activity of 25 subjects after initial immunization with canarypox–cytomegalovirus pp65. pp65-Specific lysis at an effector-to-target (E:T) ratio of 25:1 (E:T of 30:1 for subjects 9, 27, 32, 33, and 38 at month 5). pp65-Specific lysis was considered to be significant at 10%.

Table 2. pp65-Specific cytotoxic T lymphocyte precursor (CTLp) frequencies in healthy volunteers after vaccination with canarypox–cytomegalovirus phosphoprotein 65 (CMV pp65).

Immune status, donor[a]	Time after first immunization, month	CTLp/10^6 PBMC
Seronegative canarypox–CMV pp65 vaccinated		
27	7	257
15	12	105
22	12	61
29	12	147
Seronegative placebo inoculated		
25	12	UND
Naturally seropositive nonvaccinated		
16		155
H-35		206

NOTE. PBMC, peripheral blood mononuclear cells; UND, undetectable.

[a] Freshly obtained (donors H-35, 27, 15, 22, and 29) or cryopreserved (donor 16) peripheral blood PBMC were used in limiting dilution assays, as described in Materials and Methods.

57% of placebo recipients developed local pain and tenderness. Among all systemic symptoms, only headache ($P = .049$) and myalgia ($P = .047$) occurred significantly more frequently in vaccinees. Although there were other reactions among both placebo and vaccine recipients, none were of statistical significance.

Fourteen subjects reported 22 adverse events during the study. Only 2 were considered to be related to vaccination (both among seronegative canarypox–CMV pp65 recipients): a severe local reaction after dose 2 (subject was discontinued from the study) and 35 days of rash (contact dermatitis) after dose 3 in another subject. Two serious (nonrelated) adverse events (orthopedic) were reported, each requiring hospitalization.

Induction of pp65-specific CTL responses in all 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 CMV-seronegative subjects were CTL negative at time 0, and the placebo recipients remained CTL negative, whereas all subjects who received the canarypox–CMV pp65 vaccine responded with pp65-specific CTL activity at each time point tested (figure 1). All 8 seronegative vaccinees tested after 2 doses of vaccine exhibited HLA class I–restricted pp65-specific CTL activity; after inoculation 3, 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 month 26. We tested 1–4 of the naturally seropositive subjects at months 3, 4, 7, 12, and 26: all were pp65-specific CTL-positive and remained positive for the entire study period. In the originally seronegative vaccinees, specific lysis was obtained even at very low E:T ratios (1.5:1 or 3:1) in 13 of the 14 volunteers at 1–3 time points after vaccination and at an E:T ratio of 6:1 in 9 volunteers at all time points from months 4 to 12. Of the 14 originally seronegative vaccinees, 13 exhibited ⩾1 HLA alleles (A1, A2, A3, A68, B7, and B35) previously shown to present pp65 epitopes; 1 vaccinee had alleles (A31, B49, and B15) not previously shown to present pp65. All of these subjects mounted pp65-specific CTL responses after vaccination. These results indicate that CMV pp65, as expressed by the ALVAC recombinant, induced CTL responses in persons with different HLA haplotypes, that 2 doses of canarypox–CMV pp65 were sufficient for CTL induction, and that CTL activity did not change significantly during the 20 months after the last vaccine dose in the 5 subjects tested at month 26.

Similarity of pp65-specific CTLp frequencies in canarypox–CMV pp65 vaccinees and naturally seropositive persons. CTLp frequencies were determined at months 7 or 12 (1 and 6 months after inoculation 4) 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 frequencies per 10^6 PBMC was similar to that of the 2 naturally seropositive donors (range, 105–257/10^6 PBMC for vaccinees 27, 15, and 29 vs. 155 and 206/10^6 PBMC for naturally seropositive subjects), whereas, in the fourth subject, it was lower (61; table 2). CTLp frequencies were undetectable in volunteer 25, a seronegative subject inoculated with placebo. In an experiment to test the number of CTLp frequencies in the course of the immunization process, PBMC cryopreserved at 0, 4, and 7 months from vaccinee 35 were used as effector cells. CTLp frequencies were undetectable at month 0, were not tested after 1 or 2 doses, and were detected after 3 vaccinations (92/10^6 PBMC at month 4) and after 4 vaccinations (264/10^6 PBMC at month 7). These results indicate

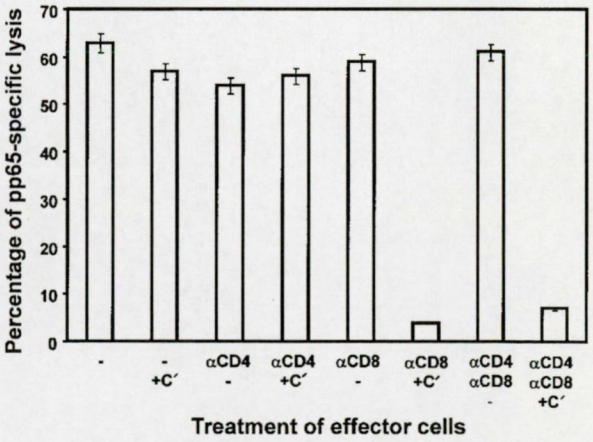

Figure 2. Phenotype of phosphoprotein 65 (pp65)–specific effector cells. Bulk cytotoxic T cell (CTL) cultures were established from fresh peripheral blood mononuclear cells of donor 15. After 11-day culture in vitro, effector cells were harvested, and aliquots of 3×10^6 cells were depleted of CD4 lymphocytes by anti-CD4 monoclonal antibody (MAb) plus complement (+C′) or of CD8 lymphocytes by anti-CD8 MAb + C′ or of both CD4 and CD8 by both antibodies + C′. pp65-Specific lysis is shown at an effector-to-target ratio of 30:1.

Table 3. Lymphocyte proliferation responses in healthy volunteers after canarypox–cytomegalovirus phosphoprotein 65 (CMV pp65) vaccination.

Vaccine group	pp65-Specific proliferation by month						
	0	3	4	6	7	12	26
Seronegative canarypox–CMV pp65 vaccinated	1.53 ± 0.63 (0/14)	11.04 ± 12.6 (9/14)	16.6 ± 17.5 (13/14)	12.1 ± 7.1 (12/14)	22.1 ± 26.5 (11/12)	19.02 ± 26.1 (13/14)	24.8 ± 29.9 (4/5)
Seronegative placebo inoculated	0.93 ± 0.25 (0/7)	1.78 ± 1.4 (0/7)	1.25 ± 0.46 (0/6)	1.6 ± 0.6 (0/7)	1.4 ± 0.51 (0/6)	2.6 ± 0.82 (0/6)	1.7 ± 0.98 (0/2)
Seropositive canarypox–CMV pp65 vaccinated	26.03 ± 21.87 (2/2)	28.35 ± 13.1 (4/4)	13.1 ± 11.1 (4/4)	7.4 ± 1.4 (2/2)	12.1 ± 0.94 (3/3)	62.7 ± 26.7 (3/3)	22.9 ± 23.4 (2/2)

NOTE. Data are mean stimulation index (SI) ± SD (no. of subjects positive/total no. of subjects tested) by months after immunization.

that the number of memory CTL increased during the repeated immunization of this subject with canarypox–CMV pp65. Furthermore, CTLp frequencies detected at 7–12 months after the first immunization with the canarypox–CMV pp65 vaccine in seronegative persons were similar to that detected in CMV-seropositive subjects after natural infection that occurred at an undefined time in the past and that may have been boosted or expanded by multiple exposures.

Phenotype of pp65-specific CTL. The phenotype of pp65-specific CTL was determined at month 5 in originally seronegative subjects who received the canarypox–CMV pp65 vaccine. Figure 2 shows the results of a depletion experiment that used cells from vaccinee 15. Although depletion of effector cells with anti-CD4 antibodies and C′ did not change the percentage of 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%, which indicates that the effectors were $CD8^+$. Of 13 vaccinees analyzed for phenotype of pp65-specific CTL, 11 exhibited $CD8^+$ effector cells. In 2 vaccinees (subjects 38 and 10), the determination of CTL phenotypes did not give clear results because of low pp65-specific CTL activity (subject 38 had 16% of pp65-specific lysis at an E:T ratio of 30:1 and 9% of pp65-specific lysis at an E:T ratio of 15:1) and the apparent presence of effector cells partially resistant to CD4 and CD8 antibodies and C′ (subject 10).

pp65-specific lymphoproliferative responses in vaccinees. To determine whether pp65, as expressed by canarypox–CMV pp65, elicits helper T lymphocytes, we analyzed pp65-specific lymphocyte proliferation responses. Responses to a purified pp65 preparation were measured for all subjects (table 3). In the originally seronegative subjects 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 2 immunizations. All of the vaccinated subjects showed pp65-specific lymphocyte proliferation: 7 were positive at all time points tested, and 7 were positive at 2–4 time points (months 3–12). Four of the 5 vaccinated subjects tested at month 26 were found to be positive (table 3). Originally seronegative persons who received the placebo remained negative. Originally seropositive vaccinees remained positive, although there was some decrease in lymphocyte proliferation indices after 3 inoculations. By month 12, SIs were greater than before the initial immunization process (table 3).

Antibody responses in vaccinees. Antibodies were measured by a pp65-specific ELISA in 21 volunteers at different times 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 (table 4). Originally seronegative vaccinees who were inoculated with placebo remained negative.

Discussion

This ALVAC construct was safe and well tolerated in most subjects, which confirms the safety profile observed with all ALVAC recombinant vaccines tested in human volunteers. CMV pp65 is a major CTL target in naturally seropositive persons [16–19, 22, 23], and CTL induction in humans was demonstrated after Towne attenuated CMV vaccination in 3 of 4 immunized volunteers for 6 months [38], although the target protein(s) specificity of these CTL was not determined. In the present trial, we showed for the first time that pp65-specific CTL can be induced in humans by a recombinant vaccine candidate. Canarypox–CMV pp65 induced $CD8^+$ CTL responses in all of the seronegative subjects, and the responses were detectable at each time point between months 3 and 12 and during a 26-month observation period in all 5 persons tested. Moreover, the CTLp frequency was comparable with that of unvaccinated naturally CMV-seropositive donors, and CTL responses were accompanied by CMV pp65–specific lymphocyte proliferation and antibody responses. All volunteers who were tested after the second of 4 immunizations were already positive for pp65-specific CTL, which suggests that 2 immunizations may be sufficient for induction of a CTL response. Tests were not done after a single vaccination. The possibility that the pp65-specific CTL responses of the originally seronegative subjects reflected natural intercurrent CMV infection during the study period was ruled out by the absence of CMV gB-specific ELISA antibodies in these persons (data not shown).

We observed pp65-specific lysis of target cells in bulk CTL assays at low E:T ratios (≤6:1) in most subjects at each time point, which suggests that pp65 is a strong CTL inducer in humans when expressed by the canarypox–CMV pp65 recombinant and that CTL responses do not differ significantly among per-

Table 4. Induction of phosphoprotein 65 (pp65)–specific antibodies in vaccinees by canarypox–cytomegalovirus (CMV) pp65.

Subject group	pp65-Specific OD of serum samples after immunization 1 by month				
	0	3	4	6	7
Seronegative canarypox–CMV pp65 vaccinated	−0.004 ± 0.029	0.077 ± 0.041	1.261 ± 0.771	1.047 ± 0.452	0.9818 ± 0.29
Seronegative placebo inoculated	−0.014 ± 0.035	−0.031 ± 0.106	−0.003 ± 0.062	0.028 ± 0.028	0.017 ± 0.029
Seropositive canarypox–CMV pp65 vaccinated	1.862 ± 1.608	ND	ND	1.808 ± 1.685	1.843 ± 1.635

NOTE. Data are mean OD ± SD. In the group of originally seronegative vaccinees, serum samples of 6–8 subjects at a dilution of 1:200 were tested at indicated time points. Cutoff, mean OD of serum 200 × dilution + 2 × SD of originally seronegative subjects at month 0:0.053. ND, not done; OD, optical density.

sons. The HLA haplotype of the vaccinees was determined before the trial, to guide selection of mismatched targets in the CTL assays, but vaccinees were not selected for the known HLA haplotypes presenting pp65. CD8 T cell reactivity to pp65 is strongly linked to HLA-A2 [39, 40]. A1, A3, A68, B7, and B35 also were shown to present pp65 epitopes [18, 19, 23]. Among the canarypox–CMV pp65 vaccinees, 57% were positive for A2, and 93% were positive for ⩾1 pp65-presenting alleles. One vaccinee (subject 31) had HLA haplotype with an allele composition not previously shown to present pp65 (A31, B49, and B15). Therefore, our findings indicate that the range of pp65-presenting alleles is even broader than that previously shown.

Restriction maps of DNA extracted from laboratory strains and fresh isolates of human CMV show that each strain is distinct but related [41–45]. 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 may be associated with certain CMV diseases [46–50]. The *pp65* gene of the canarypox–CMV pp65 recombinant was derived from CMV Towne strain. It has not been demonstrated that the pp65-specific CTL induced by the canarypox–CMV pp65 recombinant would recognize target cells expressing pp65 from other strains in naturally infected persons. However, the use of the same canarypox–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 subjects tested (i.e., memory CTL from persons of heterologous HLA haplotypes responded to the canarypox–CMV pp65 recombinant [23]), which indicates common CTL epitopes in the Towne strain and in the clinical strains that infected these persons.

Our results do not provide direct evidence for the protective effect of canarypox–CMV pp65 against CMV disease. However, the pp65-specific lysis at low E:T ratios in the bulk CTL assays and the similar CTLp frequencies of PBMC from vaccinees and from naturally seropositive persons suggest that immunization with canarypox–CMV pp65 might elicit a sufficient immunologic response to confer protection similar to that obtained through natural infection. Although the phenotype of pp65-specific CTL in some naturally seropositive persons is both $CD8^+$ and $CD4^+$ mediated [23], the CTL phenotype in all 11 vaccinees who could be tested was $CD8^+$. Lymphocyte proliferation responses specific for pp65 detected in our in vitro assays indicate that the canarypox–CMV pp65 recombinant also stimulates $CD4^+$ T cells. This suggests the release of cytokines that could contribute to the protective effect of the vaccine. In murine models, CD4 helper function, although not required for $CD8^+$ CTL generation, is needed for persistence of $CD8^+$ cell memory [51, 52].

The function of the anti–pp65-specific antibodies is uncertain, but their induction by the canarypox–CMV pp65 reflects stimulation of B cells by the insert of the ALVAC recombinant. The demonstration of an antibody response to pp65 may be useful in future vaccine studies as a surrogate for CTL response. This clinical trial clearly shows that CMV pp65, when expressed by an ALVAC recombinant, induces strong $CD8^+$ CTL responses in humans. Thus, we conclude that ALVAC CMV recombinants can stimulate functional immune responses and are promising candidates for immunization against CMV disease, perhaps when combined with antigens that stimulate neutralizing antibodies.

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References

1. Stagno S, Pass RF, Sworsky ME, et al. Congenital cytomegalovirus infection: the relative importance of primary and recurrent maternal infection. N Engl J Med 1982;306:945–9.
2. Fowler KB, Stagno S, Pass RF, Britt WJ, Boll TJ, Alford CA. The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. N Engl J Med 1992;326:663–7.
3. Boppana SB, Fowler KB, Britt WJ, Stagno S, Pass RF. Symptomatic congenital

cytomegalovirus infection in infants born to mothers with preexisting immunity to cytomegalovirus. Pediatrics 1999;104:55–60.

4. Britt WJ, Alford CA. Cytomegalovirus. In: Fields BN, Knipe DM, Howley PM, et al., eds. Fields virology. Philadelphia: Lippincott-Raven, 1996; 2493–523.
5. Chou S, Norman DJ. Effect of OKT3 antibody therapy on cytomegalovirus reactivation in renal transplant recipients. Transplant Proc 1985;17:2755–6.
6. Singh N, Dummer JS, Ho M, et al. Infections with cytomegalovirus and other herpesviruses in 121 liver transplant recipients: transmission by donated organ and the effects of OKT3 antibodies. J Infect Dis 1988;158: 124–31.
7. Quinnan GV, Kirmani N, Rook AH, et al. Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non–T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. N Engl J Med 1982;307:7–13.
8. Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T lymphocyte response to cytomegalovirus following human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood 1991;78:1373–80.
9. Reusser P, Attenhofer R, Hebart H, Helg C, Chapuis B, Einsele H. Cytomegalovirus-specific T-cell immunity in recipients of autologous peripheral blood stem cell or bone marrow transplants. Blood 1997;89:3873–9.
10. 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.
11. Riddell SR, Gilbert MJ, Li CR, Walter BA, Greenberg PD. Reconstitution of protective $CD8^+$ cytotoxic T lymphocyte responses to human cytomegalovirus in immunodeficient humans by the adoptive transfer of T cell clones. In: Michelson S, Plotkin SA, eds. Multidisciplinary approach to understanding cytomegalovirus disease. Amsterdam: Elsevier Science, 1993:155–64.
12. Li CR, Greenberg PD, Gilbert MJ, Goodrich JM, Riddell SR. Recovery of HLA-restricted cytomegalovirus (CMV)–specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood 1994;83:1971–9.
13. 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.
14. Borysiewicz LK, Hickling JK, Graham S, et al. Human cytomegalovirus-specific cytotoxic T cells. J Exp Med 1988;168:919–31.
15. Alp NJ, Allport TD, Van-Zanten J, Rodgers B, Sissons JGP, Borysiewicz LK. Fine specificity of cellular immune responses in humans to human cytomegalovirus immediate-early 1 protein. J Virol 1991;65:4812–20.
16. Gilbert MJ, Riddell SR, Li CR, Greenberg PD. Selective interference with class I major histocompatibility complex presentation of the major immediate-early protein following infection with human cytomegalovirus. J Virol 1993;67:3461–9.
17. Riddell SR, Rabin M, Geballe AP, Britt WJ, Greenberg PD. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. J Immunol 1991;146:2795–804.
18. McLaughlin-Taylor E, Pande H, Forman S, et al. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for $CD8^+$ virus-specific cytotoxic T lymphocytes. J Med Virol 1994;43:103–10.
19. Wills MR, Carmichael AJ, Mynard K, et al. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J Virol 1996;70:7569–79.
20. Hopkins JI, Fiander AN, Evans AS, Delchambre M, Gheysen D, Borysiewicz LK. Cytotoxic T cell immunity to human cytomegalovirus glycoprotein B. J Med Virol 1996;49:124–31.
21. Boppana SB, Britt WJ. Recognition of human cytomegalovirus gene products by HCMV-specific cytotoxic T cells. Virology 1996;222:293–6.
22. Kern F, Surel IP, Faulhaber N, et al. Target structures of the $CD8^+$–T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. J Virol 1999;73:8179–84.
23. Gyulai Z, Endresz V, Burian K, et al. Cytotoxic T lymphocyte (CTL) responses to HCMV pp65, IE1 exon 4, gB, pp150, and pp28 in healthy seropositive individuals: reevaluation of prevalence of IE-specific CTL. J Infect Dis 2000;181:1537–46.
24. Baxby D, Paoletti E. Potential use of nonreplicating vectors as recombinant vaccines. Vaccine 1992;10:8–9.
25. Tartaglia J, Perkus ME, Taylor J, et al. NYVAC: a highly attenuated strain of vaccinia virus. Virology 1992;188:217–32.
26. Plotkin SA, Cadoz M, Meignier B, et al. The safety and use of canarypox vectored vaccines. Dev Biol Stand 1995;84:165–70.
27. Cadoz M, Strady A, Meignier B, et al. Immunization with canarypox virus expressing rabies glycoprotein. Lancet 1992;339:1429–32.
28. Fries LF, Tartaglia J, Taylor J, et al. Human safety and immunogenicity of a canarypox-rabies glycoprotein recombinant vaccine: an alternative poxvirus vector system. Vaccine 1996;14:428–34.
29. Taylor J, Weinberg R, Tartaglia J, et al. Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. Virology 1992;187:321–8.
30. Pialoux G, Excler JL, Riviere Y, et al. A prime-boost approach to HIV preventive vaccine using a recombinant canarypox virus expressing glycoprotein 160 (MN) followed by a recombinant glycoprotein 160 (MN/LAI). AIDS Res Hum Retroviruses 1995;11:373–81.
31. Clements-Mann ML, Weinhold K, Matthews TJ, et al. Immune responses to human immunodeficiency virus (HIV) type 1 induced by canarypox expressing $HIV\text{-}1_{MN}$ gp120, $HIV\text{-}1_{SF2}$ recombinant gp120, or both vaccines in seronegative adults. J Infect Dis 1998;177:1230–46.
32. Evans TG, Keefer MC, Weinhold KJ, et al. A canarypox vaccine expressing multiple human immunodeficiency virus type 1 genes given alone or with Rgp120 elicits broad and durable $CD8^+$ cytotoxic T lymphocyte responses in seronegative volunteers. J Infect Dis 1999;180:290–8.
33. 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) in humans. J Infect Dis 1999;180: 843–6.
34. Gonczol E, Furlini G, Ianacone J, Plotkin SA. A rapid microneutralization assay for cytomegalovirus. J Virol Methods 1986;14:37–41.
35. Lefkovits I, Waldmann H. Limiting dilution analysis of cells of the immune system. Cambridge, UK: Cambridge University Press, 1979:60–82.
36. Pande H, Lee TD, Churchill MA, Zaia JA. Structural analysis of a 64-kDa major structural protein of human cytomegalovirus (Towne): identification of a phosphorylation site and comparison to pp65 of HCMV (AD169). Virology 1990;178:6–14.
37. Endresz V, Kari L, Berencsi K, et al. 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;17:50–8.
38. Adler SP, Hempfling SH, Starr SE, Plotkin SA, Riddell S. Safety and immunogenicity of the Towne strain cytomegalovirus vaccine. Pediatr Infect Dis J 1998;17:200–6.
39. Diamond DJ, York J, Sun JY, Wright CL, Forman SJ. Development of a candidate HLA A*0201–restricted peptide-based vaccine against human cytomegalovirus infection. Blood 1997;90:1751–67.
40. Kern F, Faulhaber N, Frömmel C, et al. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. Eur J Immunol 2000;30:1676–82.
41. Chandler SH, McDougall JK. Comparison of restriction site polymorphisms among clinical isolates and laboratory strains of human cytomegalovirus. J Gen Virol 1986;67:2179–92.
42. Greenaway PJ, Oram JD, Downing RG, Patel K. Human cytomegalovirus DNA: *Bam*HI, *Eco*RI, and *Pst*I restriction endonuclease cleavage maps. Gene 1982;18:355–60.
43. Spector DH, Hock L, Tamashiro JC. Cleavage maps for human cytomega-

lovirus DNA strain AD169 for restriction endonucleases *Eco*RI, *Bg*III, and *Hind*III. J Virol 1982;42:558–82.

44. Weststrate MW, Geelen JL, van der Noordaa J. Human cytomegalovirus DNA: physical maps for restriction endonucleases *Bg*III, *Hind*III, and *Xba*I. J Gen Virol 1980;49:1–21.
45. Weststrate MW, Geelen JL, Wertheim PM, van der Noordaa J. Comparison of the physical maps of the DNAs of two cytomegalovirus strains. J Gen Virol 1983;64:47–55.
46. Fries BC, Chou S, Boeckh M, Torok-Storb B. Frequency distribution of cytomegalovirus envelope glycoprotein genotypes in bone marrow transplant recipients. J Infect Dis 1994;169:769–74.
47. Shepp DH, Match ME, Ashraf AB, Lipson SM, Millan C, Pergolezi R. Cytomegalovirus glycoprotein B groups associated with retinitis in AIDS. J Infect Dis 1996;174:184–7.
48. Vogelberg C, Meyer-König U, Hufert FT, Kirste G, von Laer D. Human cytomegalovirus glycoprotein B genotypes in renal transplant recipients. J Med Virol 1996;50:31–4.
49. Rasmussen L, Hong C, Zipeto D, et al. Cytomegalovirus gB genotype distribution differs in human immunodeficiency virus–infected patients and immunocompromised allograft recipients. J Infect Dis 1997;175:179–84.
50. Chern KC, Chandler DB, Martin DF, Kuppermann BD, Wolitz RA, Margolis TP. Glycoprotein B subtyping of cytomegalovirus (CMV) in the vitreous of patients with AIDS and CMV retinitis. J Infect Dis 1998;178:1149–53.
51. Sauzet JP, Gras-Masse H, Guillet JG, Comard E. Influence of strong CD4 epitope on long-term virus-specific cytotoxic T cell responses induced in vivo with peptides. Int Immunol 1996;8:457–65.
52. Rahemtulla A, Fung-Leung WP, Schilham MW, et al. Normal development and function of $CD8^+$ cells but markedly decreased helper cell activity in mice lacking CD4. Nature 1991;353:180–4.

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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 (UL123) induce a cytotoxic T-cell response

Klara Berencsi*, Eva Gönczöl*, Valeria Endresz*, John Kough†, Shin Takeda‡, Zsofia Gyulay*, Stanley A. Plotkin§ and Robert F. Rando¶||

We reported earlier that an adenovirus (Ad) recombinant expressing the full-length human cytomegalovirus (HCMV) glycoprotein B (gB) gene induces gB-specific cytotoxic T lymphocyte (CTL) responses in CBA (H-2^k) mice (Berencsi et al., J. Gen. Virol. 74, 257–2512, 1993). *Here we show that mice immunized with Ad recombinant viruses expressing truncated forms of the gB gene containing the first 700 (Ad-700), 465 (Ad-465) or 303 (Ad-303) amino acids of gB or an Ad construct containing exon 4 (E4) of the HCMV immediate early 1 (IE1) gene (Ad-IE1 (E4)) demonstrate HCMV-specific CTL responses. These data suggest the importance of the first 303 amino acids of the gB polypeptide and the IE1 E4 product in designing a vaccine to induce anti-HCMV CTL responses. Copyright © 1996 Elsevier Science Ltd.*

Keywords: CTL epitope; HCMV; IE1 exon 4; Ad-HCMV recombinants

Infections with human cytomegalovirus (HCMV) are common and usually asymptomatic; however, the incidence and spectrum of HCMV-induced disease in newborns and immunocompromised hosts, including organ transplant recipients, establish this virus as an important human pathogen[1–3]. In addition, HCMV has been associated with the development of atherosclerosis[4] and restenosis after coronary angioplasty[5].

Although the exact role of individual HCMV proteins in protective immunity in humans is unclear, the viral surface glycoprotein B (gB, UL55), glycoprotein H (gH, UL75), the major tegument proteins pp65 (UL83) and pp150 (UL32), the major immediate early proteins (IE1, UL123 and IE2, UL122) as well as UL69 of the virus, appear to be important in the neutralizing antibody (NA), lymphocyte proliferation and cytotoxic T lymphocyte (CTL) responses to the virus after natural infection[6–17]. The majority of neutralizing epitopes on gB are in the C-terminal part of the molecule, while the N-terminal part is considered less important in inducing antibody response[18,19]. Recently, we demonstrated that purified and subcutaneously inoculated gB induces NA and lymphocyte proliferation responses in humans[20], and NA, lymphocyte proliferation and CTL responses when expressed by adenovirus (Ad) or poxvirus recombinants in mice and guinea pigs[10,21–23]. However, the CTL epitopes of gB have not been precisely localized. To date, mapping of CTL epitopes on gB has indicated that the N-terminal 513 amino acids and a region between amino acids 619 and 628 can be targets of CTL recognition in certain seropositive individuals[11,24]. As for mapping of CTL epitopes of the IE protein, CTL from one individual specifically lysed target cells sensitized with a peptide spanning amino acid residues 162–180 of the exon 4 (E4) region of the IE1 polypeptide[25].

In the present study, we asked whether the N-terminal part of gB is important in eliciting CTL responses, and analyzed the CTL responses induced by Ad recombinants expressing truncated forms of the HCMV-gB. We also asked whether the E4 protein, when expressed by a recombinant virus *in vivo*, can induce a CTL response, in order to confirm its immunogenicity in a potential combined vaccine against HCMV infection.

*The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA. †United States Environmental Protection Agency (USEPA), 401 M Street S. W., Washington DC 20460, USA. ‡Department of Surgery II, Nagoya University School of Medicine, 65 Tsurumai-cho Showa-ku, Nagoya 466, Japan. §Pasteur Merieux Connaught, 3 Avenue Pasteur, Marnes la Coquette 92430, France. ¶NIH-NIDR, Bethesda, MD, USA. ||To whom correspondence should be addressed at: Aronex Pharmaceutical Inc., 9391 Grogan's Mill Road, The Woodlands, TX 77380, USA. (Received 17 April 1995; revised 17 October 1995; accepted 17 October 1995)

MATERIALS AND METHODS

Viruses and cells

The early region 3 (E3)-deleted Ad-5 mutant virus which lacks the *Xba*I D fragment of Ad (Ad5ΔE3), the Ad-gB recombinant, and the replication of Ad-gB in the mouse lung have been described[22,26,27]. Ad recombinants expressing truncated forms of gB or E4 of the IE1 gene were constructed by overlap recombination using plasmid pAd-5 (m.u. 0–75.9), and the corresponding plasmids containing the subfragments of the gB or IE genes inserted into the E3-deleted plasmid pAd-5 (m.u. 59–100) as described[26]. Truncated gB and IE1 gene fragments were constructed by amplification of plasmids containing the intact gB (pAd-5-gB)[26] or IE1 (pRL43a)[28] genes, using the polymerase chain reaction (PCR) technique. The 5′ gB- and IE1-specific PCR primers were engineered so that an *Xba*I site was placed upstream of the initial ATG codon. The 5′ IE1 primer was also engineered to change the first codon in the E4 region to an ATG, resulting in methionine as the first residue of the translated protein product[29]. The sequences of the 5′ gB and IE1 PCR primers are 5′-acacgcaagagatctagacgcgcctcat-3′ and 5′-ttatcctcctcta gaatgaaacagattaag-3′, respectively. The 3′ gB-specific primers were engineered to place a termination signal just downstream of the indicated amino acid of the gB gene[30]. The sequences of the 3′ gB-specific primers used to generate Ad-700, Ad-465 and Ad-303 are 5′-tcgtccagactctagaggtagggc-3′, 5′-cgactccattctagattaatgag ttgc att-3′, 5′-caaagtcggagtctagagtctagttcggaaa-3′ and 5′-cagataagtggtctagatctaagcgtagctacg-3′, respectively. The sequence of the 3′ IE1 PCR primer is 5′-atatatatattctag agtttactggtcagc-3′[29]. *Figure 1* shows schematic diagrams of the Ad-HCMV recombinant viruses.

Expression of gB-polypeptides was detected in A549 human lung carcinoma cells infected with Ad-700, Ad-465 or Ad-303 recombinants by an immunofluorescence test using monoclonal antibody (Mab) 3C2 (courtesy of R.C. Gehrz, Biomedical Research Center, St. Paul, MN, USA)[13,31]. This Mab recognizes an epitope in the region of the gB protein between amino acids 50 and 77. Expression of the E4 polypeptide in A549 cells infected with Ad-IE1 (E4) was confirmed by immunofluorescence assay using Mab P63-27 (kindly provided by W. Britt, University of Alabama at Birmingham, AL, USA).

The parental Ad strain Ad-5, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and recombinant Ad were grown and titered on A549 cells. For immunization of mice, Ad grown on A549 cells was purified by CsCl gradient centrifugation[26].

A vaccinia (WR strain) recombinant carrying the IE1 gene of HCMV (Vac-IE1) and the parental vaccinia (WT-Vac) were provided by Enzo Paoletti (Virogenetics Corp., Troy, NY, USA) and used for infection of target cells in CTL assays for Ad-IE1 (E4). The vaccinia recombinant carrying the gB gene of HCMV (Vac-gB) has been described[10].

Immunization of mice

Six- to 8-week-old female CBA mice of MHC haplotype H-2^k (Jackson Laboratories, Bar Harbor, ME, USA) were inoculated intraperitoneally with 10^8 p.f.u. of recombinant viruses or as a control with Ad5ΔE3 parental virus; sera and splenocytes were collected and analyzed for antibody and CTL responses.

Neutralization assay

HCMV (Towne strain) neutralization titers were determined on MRC-5 cells by microneutralization and plaque-reduction neutralization assays[32]. Ad-5 neutralization titers were detected by a microneutralization assay as described for HCMV[32], except that A549 instead of MRC-5 cells were used.

ELISA assay

An ELISA assay was carried out as described[33]. As antigens, HCMV (Towne strain) was collected by high-speed centrifugation from culture medium of infected MRC-5 cells, and as control, lysates of uninfected MRC-5 cells, both at a protein concentration of 1.5 μg $well^{-1}$, were used. Peroxidase-conjugated goat affinity-purified $F(ab')_2$ fragment to mouse IgG (Cappel, Durham, NC, USA) was used as a second antibody. The optical density (O.D.) was read by a microtiter plate reader (Bio-Tek Instruments, VT, USA) at 490 nm. Antibody response was considered positive when the O.D. value exceeded the mean density value of Ad5ΔE3-immunized mice plus two standard deviations.

CTL assay

CTL assay and the characterization of cells responsible for insert-specific lysis were carried out as described[22]. Briefly, spleens of CBA mice immunized with the Ad-gB or truncated-gB recombinant viruses or Ad5ΔE3 were aseptically removed and cell suspensions were prepared from them. Cells were suspended at 2.5×10^6 viable cells ml^{-1} in RPMI 1640 medium containing 5% fetal bovine serum, 2×10^{-5} M 2-mercaptoethanol, 10 mM-HEPES buffer, 2 mM-glutamine and 50 μg ml^{-1} gentamicin. Spleen cell cultures were restimulated *in vitro* with Ad-gB-infected (multiplicity of infection (m.o.i) 10) autologous spleen cells (effector:stimulator ratio 2:1) for 5 days in 24-well plates. Cytolytic activity of non-adherent spleen cells was tested in a ^{51}Cr release assay. Target cells (L-929 and MC57) were infected with the Ad5ΔE3 (m.o.i. 40–80, for 40 h) or with Vac-gB, Vac-IE1 or WT-Vac (m.o.i. 5–10, for 4 h). Cells were labeled with 100 mCi of [^{51}Cr]Na_2CrO_4 (Amersham, Arlington Heights, IL, USA; specific activity 250–500 mCi mg^{-1}) for 1 h. Labeled target cells were mixed with effector cells at various effector:target ratios in triplicate using 96-well U-bottomed microtiter plates and incubated for 4 h. Percentage specific ^{51}Cr release was calculated as [(c.p.m. experimental release − c.p.m. spontaneous release)/(c.p.m. maximal release − c.p.m. spontaneous release)] × 100. Standard deviation of the mean of triplicate culture was <10%, and spontaneous release was always <25%. Significance of lysis was determined by comparing test c.p.m. to control c.p.m. using the Student's *t*-test.

To characterize the cells responsible for anti-gB cytotoxicity, 3×10^6 *in vitro* restimulated spleen cells of Ad-recombinant-immunized mice were incubated with anti-mouse CD4 Mab (Pharmingen, San Diego, CA,

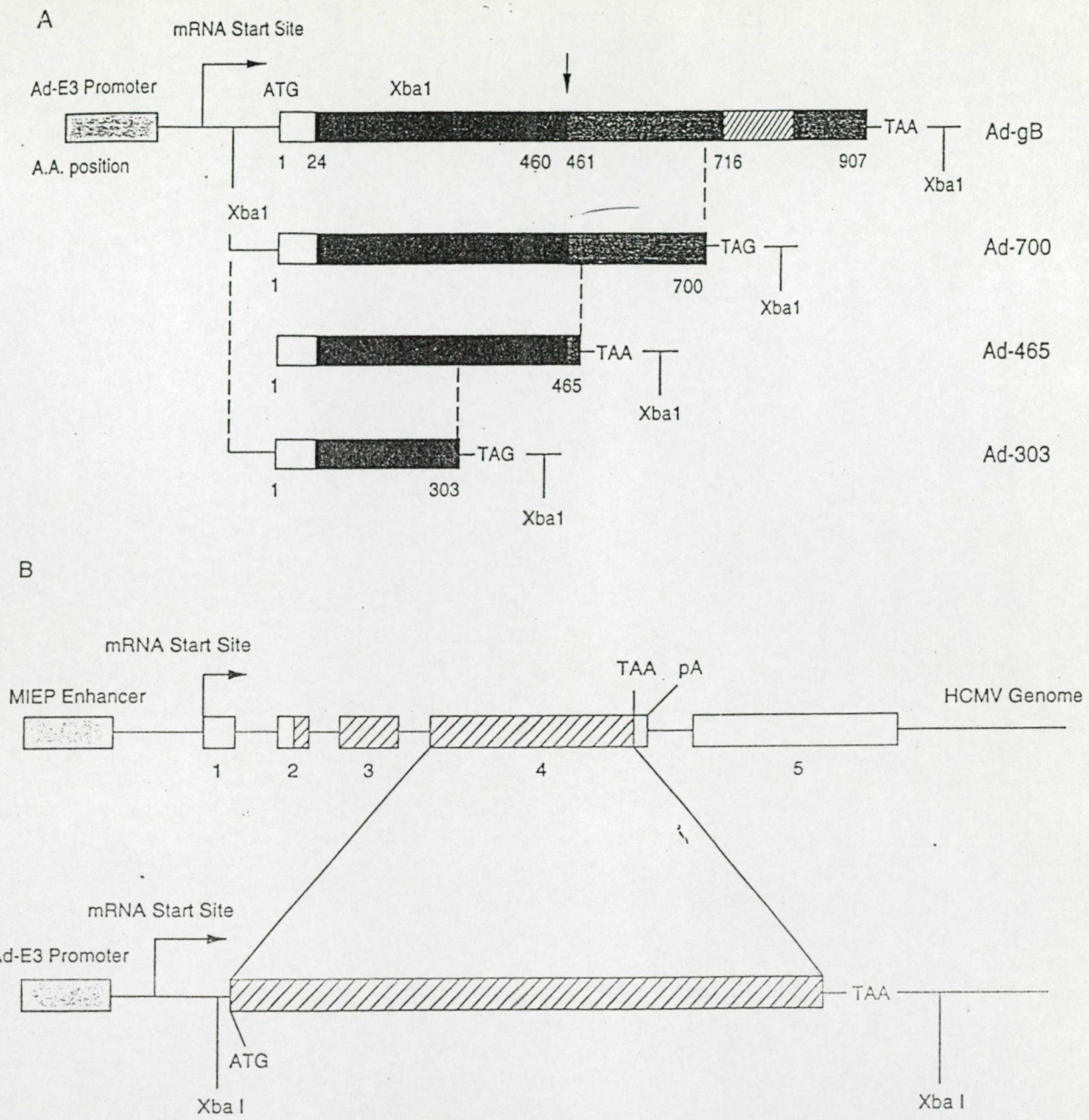

Figure 1 Schematic representation of the HCMV gene fragments inserted into the Ad E3 deletion vector. (A) Full-length and truncated versions of the HCMV-gB gene are depicted as *Xba*I DNA fragments inserted downstream of the Ad E3 promoter. The gB open reading frame starting at the first methionine (ATG; amino acid position 1) and the site of proteolytic processing of the gB gene (arrow between amino acids 460 and 461) are indicated. Also indicated are the gB leader peptide, amino acids 1–24 (□), N-terminal part (◇), C-terminal part (shaded square), transmembrane region (hatched square) and the positions of the *Xba*I sites. (B) The E4 region of the major immediate early protein (MIEP) locus was amplified using PCR and inserted downstream of the Ad E3 promoter. The PCR primers were engineered so that a methionine (ATG) codon begins E4. In addition, a redundant termination signal was placed downstream from the natural stop codon. The positions for the initiation of transcription and of the *Xba*I sites are indicated. The first four exons of the HCMV MIEP complex are part of the mature mRNA for the HCMV IE1 protein. Striped boxes indicate the actual portions of these exons which encode the IE1 protein

USA; Cat. No. 3:01061D; 20 μg/3 × 10^6 cells) or CD8 Mab (Accurate, Westbury, NY; Cat No. CL-8921; diluted 1:4) for 60 min at 4°C, and further incubated in the presence of rabbit complement (Accurate; Low-Tox M; diluted 1:10) for 30 min at 37°C. Cells were washed twice and used as effector cells in a ^{51}Cr release test.

RESULTS AND DISCUSSION

Antibody responses to truncated forms of gB

Sera obtained from all immunized mice showed a significant level of Ad-5-NA. Sera obtained from mice inoculated with Ad-gB or Ad-700, but not sera from mice inoculated with the other two truncated gB recombinants (Ad-465 and Ad-303), induced a significant level of HCMV-NA (*Table 1*). These data are consistent with the well-confirmed observation that the HCMV-gB immunodominant antibody neutralizing domain is located between amino acids 476–645 of the gB gene[15,18,30]. In addition, these data show that the neutralizing domain located between amino acids 28–67[19] induced no NA response in CBA mice.

ELISAs used to confirm production of anti-HCMV antibodies in Ad recombinant-immunized mice revealed antibodies in all groups (*Table 1*). However, only 12/16

Table 1 Antibody responses to Ad-recombinants expressing truncated forms of HCMV-gB

Mice inoculated with:	Specific neutralizing[a] antibodies		
	HCMV	Ad-5	ELISA[b] HCMV
Ad-gB	84.2	362.0	1313±120
Ad-700	76.1	430.5	407±201
Ad-465	5.2	430.5	487±140
Ad-303	5.6	362.0	266±135
Ad5DE3	4.2	440.5	44±31

[a]Geometric mean of serum dilutions of eight mice in each group showing complete inhibition in the microneutralization assay. [b]Mean O.D. of sera from 10–16 mice at 490 nm±S.D. at 1:40 serum dilution

(75%) mice immunized with Ad-303 responded with antibody production, whereas all mice in groups immunized with Ad-gB, Ad-700 or Ad-465 were positive for anti-HCMV ELISA antibodies (individual data not shown). Like the variability in antibody response to Ad-303 in our study, Curtsinger *et al.*[13] have reported antibody responses to a truncated gB antigen containing amino acids 1–163 in some seropositive individuals but not in others with the same HLA class II haplotype.

CTL responses to truncated forms of gB

Because HCMV does not infect mice *in vivo* or murine cells in culture, we used an approach based on two distinct viral expression vectors, Vac and Ad, carrying the same HCMV genes or gene fragments, to determine the CTL response of mice immunized with HCMV genes[22]. This dual vector system allows immunization of mice with one vector system (Ad) and infection of target cells with the other (Vac). Consistent with the Ad-specific NA responses (*Table 1*), mice immunized with any of the recombinants carrying intact or truncated forms of the HCMV-gB gene or with Ad5ΔE3 parental strain demonstrated Ad-specific cytotoxic responses (*Figure 2*). Ad-gB-, Ad-700-, Ad-465- or Ad-303-immunized mice but not the Ad5ΔE3-immunized mice developed gB-specific cytotoxic responses; per cent specific ^{51}Cr-release of Vac-gB-infected target cells significantly ($P<0.01$ at 50:1 effector-to-target ratio) exceeded per cent specific lysis of WT-Vac-infected target cells (*Figure 2*). Infected major histocompatibility complex (MHC) class I mismatched MC57 (H-2^b) cells were not gB-specifically lysed, indicating MHC class I (H-2^k) restricted lysis of target cells. Treatment of *in vitro* restimulated spleen cells of mice immunized with the Ad-gB, Ad-700, Ad-465 or Ad-303 with anti-mouse CD8 Mab and complement[22], abolished the anti-gB activity, whereas CD4-depleted cells continued to mediate this activity (data not shown), confirming that the CD8 lymphocyte subset mediates the gB-specific lysis of the target cells. These results suggest the presence of a CTL epitope between amino acids 1–303 in the N-terminal fragment of HCMV-gB when it is expressed by Ad recombinants in CBA mice, and an authentic presentation of gB peptides by the MHC class I molecules of cells infected with the Ad-700, Ad-465 and Ad-303 recombinants.

CTL responses to IE1 E4 product

Spleen cells from five individual mice and a pool of splenocytes from two mice, obtained 7 or 10 weeks after

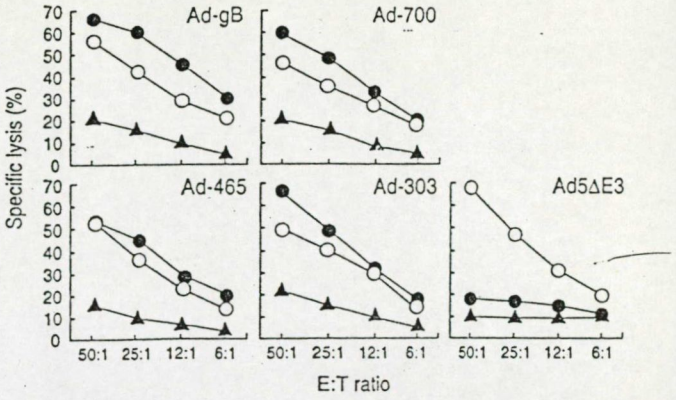

Figure 2 CTL responses in mice immunized with Ad constructs containing truncated forms of the gB gene. Ten CBA mice in each group were inoculated interperitoneally with Ad recombinants at a dose of 1×10^8 p.f.u., boosted similarly 7 weeks later, and tested for HCMV-gB-specific cytotoxicity 2 weeks after booster. Splenocytes of two mice from each group were pooled, restimulated with Ad-gB for 5 days *in vitro*, and tested in a ^{51}Cr-release assay[22]. MC57 (H-2^b, MHC-mismatched), and L929 (H-2^k, MHC-matched) cells were used as targets. Data are from a representative experiment of three performed with superimposable results. Uninfected L929 and MC57 target cells infected with Ad5ΔE3, Vac-gB or WT-Vac were not significantly lysed (not shown). L929 (H-2^k) target cells infected with Wt-Ad, ○; Vac-gB, ●; Wt-Vac, ▲

immunization, respectively, were tested for E4-specific CTL responses on Vac-IE1-infected target cells. The IE1 E4-specific lysis (per cent specific ^{51}Cr-release of Vac-IE1-infected target cells − per cent specific lysis of Wt-Vac-infected target cells) was in the range of 19–25% in each case at an effector-to-target ratio of 50:1 ($P<0.01$, Student's *t*-test), and almost no anti-IE1 activity was detected using CD8-depleted spleen cells, while CD4-depleted spleen cells retained their activity (data not shown). These results indicate a significant E4-specific stimulation of CTLs in CBA mice.

HCMV IE1 stimulates its own synthesis and cooperates with the IE2 gene product and other transactivators in the activation of gene expression[34]. The functional domains for transactivation of this protein have not been identified, and it is unknown whether the E4 product is involved in the transactivation activity of IE1. Further studies are necessary to map this activity on the IE1 protein in order to design the antigen without transactivating activity.

Precise mapping of CTL epitopes of the gB polypeptide and IE1 E4 product is a difficult task. There may be several CTL epitopes in the gB polypeptide that function depending on the MHC class I haplotype of the individual. For example, CTLs reactive with epitopes on the N-terminal 513 amino acids portion of gB were detected in one seropositive individual[11], whereas an HLA-A2.1-restricted CTL recognition has been described between amino acid positions 619–628 of HCMV-gB using CTL lines generated from two HLA-A2.1+ donors[24]. A synthetic peptide encompassing a sequence of IE1 E4 gene was identified as a class I-restricted CTL determinant using a CTL line derived from a human donor[25].

Effective protection against HCMV disease correlates with the presence of CTL in the infected individuals[35,36]. Further, treatment of HCMV infection by the adoptive transfer of HCMV-specific CTL has shown reconstitution of immunity in bone marrow transplant recipients[7]. The nature of major viral target antigens to which the CTL response is directed *in vivo* is not clear, but they

may comprise pp65 and pp150[2]. However, CD8+ CTL clones specific for gB have also been isolated from certain seropositive individuals[6]. CTL specific for IE were also detected in some HCMV-infected individuals, especially when target cells were preincubated with γ-interferon[8,37] which might upregulate expression of class I MHC genes not only *in vitro*, but after natural challenge infection. Those findings suggest the involvement of the IE protein in protective immunity.

Our data map a CTL epitope in the amino acids 1–303 region of the N-terminal portion of gB and suggest that both the C-terminal and the N-terminal portion of gB are important in inducing protective immunity and should be included in a HCMV vaccine. Our data also show that the IE E4 protein is immunogenic and induces CTL after presentation by a recombinant virus. However, the MHC-restriction of individual HCMV proteins in the heterogeneous human population is a potential obstacle in developing an effective recombinant vaccine. One approach towards overcoming this obstacle might involve the use of a combination of recombinant proteins. The gB and IE E4 gene products appear to be good candidates for combination, possibly with other HCMV gene products.

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REFERENCES

1 Alford, C.A. and Britt, W.J. Cytomegaloviruses. In: *Virology* (Eds Fields, B.N. and Knipe, D.M.). Raven Press, New York, 1990, pp. 1981–2010

2 Weller, T.H. The cytomegalovirus: ubiquitous agents with protein clinical manifestations. *N. Engl. J. Med.* 1971, 285, 203–214

3 Ho, M. Human cytomegalovirus infections in immunosuppressed patients. In: *Cytomegalovirus, Biology, and Infection: Current Topics in Infectious Disease* (Eds Greenough, III, W.B. and T. Merigan, T.). Plenum Press, New York, 1982, pp. 171–204

4 Melnick, J.L., Adams, E. and DeBakey, M.E. Human cytomegalovirus and atherogenesis. In: *Frontiers of Virology* (Eds Becker, Y., Darai, G. and Huang, E.S.). Springer, New York, 1993, pp. 80–91

5 Speir, E., Modali, R., Huang, E.-Sh. *et al.* Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. *Science* 1994, 265, 391–394

6 Riddell, S.R., Rabin, M., Geballe, A.P., Britt, W.J. and Greenberg, P.D. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. *J. Immunol.* 1991, 146, 2795–2804

7 Riddell, R.S., Gilbert, M.H., Li, C., Walter, B.A. and Greenberg, P.D. Reconstitution of protective CD8+ cytotoxic T lymphocyte responses to human cytomegalovirus in immunodeficient humans by the adoptive transfer of T cell clones. In: *Multidisciplinary Approach to Understanding Cytomegalovirus Disease* (Eds Michelson, S. and Plotkin, S.). Elsevier Science Publishers B.V., Amsterdam, 1993, pp. 155–164

8 Borysiewicz, L., Hickling, J., Graham, S., Sinclair, J., Cranage, M., Smith, G. and Sissons, J. Human cytomegalovirus specific cytotoxic T cells-relative frequency of stage specific CTL recognizing the 72 kDa immediate early protein and glycoprotein B expressed by recombinant vaccinia viruses. *J. Exp. Med.* 1988, 168, 919–931

9 Britt, W.J., Vugler, L., Butfiloski, E.J. and Stephens, E.B. Cell surface expression of human cytomegalovirus (HCMV) gp55–116 (gB): use of HCMV recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response. *J. Virol.* 1990, 64, 1079–1085

10 Gönczöl, E., deTaisne, C., Hirka, G., Berencsi, K., Lin, W., Paoletti, E. and Plotkin, S.A. High expression of human cytomegalovirus (HCMV)-gB protein in cells infected with a vaccinia-gB recombinant: the importance of the gB protein in HCMV immunity. *Vaccine* 1991, 9, 631–637

11 Liu, Y.-N.C., Klaus, A., Kari, B., Stinski, M.F., Eckhardt, J. and Gehrz, R.C. Molecular analysis of the immune response to human cytomegalovirus glycoprotein B. I. Mapping of HLA-restricted helper T cell epitopes on gp93. *J. Virol.* 1991, 65, 1644–1648

12 Liu, Y.N., Curtsinger, J., Donahue, P.R. *et al.* The N-terminal 513 amino acids of the envelope glycoprotein gB of human cytomegalovirus stimulate both B- and T-cell immune responses in humans. *J. Gen. Virol.* 1993, 74, 2207–2214

13 Curtsinger, J.M., Liu, Y.-N.C., Radeke, R. *et al.* Molecular analysis of the immune response to human cytomegalovirus glycoprotein B (gB). II. Low gB-specific T and B cell responses are associated with expression of certain HLA-DR alleles. *J. Gen. Virol.* 1994, 75, 301–307

14 Marshall, G.S., Rabalais, G.P., Stout, G.G. and Waldeyer, S.L. Antibodies to recombinant-derived glycoprotein B after natural human cytomegalovirus infection correlate with neutralizing activity. *J. Infect. Dis.* 1992, 165, 381–384

15 Pereira, L., Lennette, E., Paz, P. and Navarro, D. Humoral immunity to glycoprotein B in primary and recurrent human cytomegalovirus infections. In: *Multidisciplinary Approach to Understanding Cytomegalovirus Disease* (Eds Michelson, S. and Plotkin, S.). Elsevier Science Publishers B.V., Amsterdam, 1993, pp. 145–148

16 Rasmussen, L., Nelson, M., Neff, M. and Merigan, T.C. Jr. Characterization of two different human cytomegalovirus glycoproteins which are targets for virus neutralizing antibody. *Virology* 1988, 163, 308–318

17 Beninga, J., Kropff, B. and Mach, M. Comparative analysis of fourteen individual human cytomegalovirus proteins for helper T cell response. *J. Gen. Virol.* 1995, 76, 153–160

18 Banks, T., Huo, B., Kousoulas, K., Spaete, R., Pachi, C. and Pereira, L. A major neutralizing domain maps within the carboxyl-terminal half of the cleaved cytomegalovirus B glycoprotein. *J. Gen. Virol.* 1989, 70, 979–985

19 Qadri, I., Navarro, D., Paz, P. and Pereira, L. Assembly of conformation-dependent neutralizing domains on glycoprotein B of human cytomegalovirus. *J. Gen. Virol.* 1992, 73, 2913–2921

20 Gönczöl, E., Ianacone, J., Ho, W., Starr, S., Meignier, B. and Plotkin, S.A. Isolated gA/gB glycoprotein complex of human cytomegalovirus envelope induces humoral and cellular immune-responses in human volunteers. *Vaccine* 1990, 8, 130–136

21 Berencsi, K., Gönczöl, E., Valyi-Nagy, T. *et al.* Recombinant human cytomegalovirus (HCMV)-gB glycoprotein induces gB-specific cell-mediated immunity in mice. In: *Proceedings of the Third International Cytomegalovirus Workshop*, Bologna (Ed. Landini, M.P.). Elsevier Science Publishers, Netherlands, 1991, pp. 191–194

22 Berencsi, K., Rando, R.F., deTaisne, C., Paoletti, E., Plotkin, S.A. and Gönczöl, E. Murine cytotoxic T cell response specific for human cytomegalovirus glycoprotein B (gB) induced by adenovirus and vaccinia virus recombinants expressing gB. *J. Gen. Virol.* 1993, 74, 2507–2512

23 Gönczöl, E., Berencsi, K., Pincus, S. *et al.* Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate. *Vaccine* 1995, 13, 1080–1085

24 Utz, U., Koenig, S., Coligan, J.E. and Biddison, W.E. Presentation of three different viral peptides, HTLV-1, TAX, HCMV gB, and influenza virus M1, is determined by common structural features of the HLA-A2.1 molecule. *J. Immunol.* 1992, 149, 214–221

25 Alp, N.J., Allport, T.D., Van, Z.J., Rodgers, B., Sissons, J.G. and Borysiewicz, L.K. Fine specificity of cellular immune response in humans to human cytomegalovirus immediate–early 1 protein. *J. Virol.* 1991, 65, 4812–4820

26 Marshall, G.S., Ricciardi, R.P., Rando, R. *et al.* An adenovirus recombinant that expresses the human cytomegalovirus major

envelope glycoprotein and induces neutralizing antibodies. *J. Infect. Dis.* 1990, 162, 1177–1181

27 Berencsi, K., Uri, A., Valyi-Nagy, T. *et al.* Early region 3-replacement adenovirus recombinants are less pathogenic in cotton rats and mice than early region 3-deleted viruses. *Lab. Invest.* 1994, 71, 350–358

28 Pizzorno, M.C., O'Hare, P., Sha, L., LaTenina, R.L. and Hayward, G.S. Transactivation and autoregulation of gene expression by the immediate–early 2 gene products of human cytomegalovirus. *J. Virol.* 1988, 62, 1167–1179

29 Stenberg, R.M., Thomsen, D.R. and Stinski, M.I. Structural analysis of the major immediate–early gene of human cytomegalovirus. *J. Virol.* 1984, 49, 190–199

30 Spaete, R.R., Thayer, R.M., Probert, W.S. *et al.* Human cytomegalovirus strain Towne glycoprotein B is processed by proteolytic cleavage. *Virology* 1988, 167, 207–225

31 Kari, B., Liu, Y.N., Goertz, R., Lussenhop, N., Stinski, M.F. and Gehrz, R. Structure and composition of a family of human cytomegalovirus glycoprotein complexes designated gCI (gB). *J. Gen. Virol.* 1990, 71, 2673–2680

32 Gönczöl, E., Furlini, G., Ianacone, J. and Plotkin, S.A. A rapid microneutralization assay for cytomegalovirus. *J. Virol. Meth.* 1986, 14, 37–41

33 Hornbeck, P. Enzyme-linked immunoadsorbent assays. In: *Current Protocols in Immunology* (Eds Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Sherach, E.M. and Strober, W.). John Wiley and Sons, New York, 1991, pp. 2.1.2–2.1.22

34 Mocarski, E.S. Cytomegalovirus biology and replication. In: *The Human Herpesviruses* (Eds Roizman, B., Whitley, R.J. and Lopez, C.). Raven Press, New York, 1993, pp. 173–226

35 Quinnan, G.V.Jr., Kirmani, N., Rook, A.H. *et al.* Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. *N. Engl. J. Med.* 1982, 307, 7–13

36 Reusser, P., Riddell, S.R., Meyers, J.D. and Greenberg, P.D. T-lymphocyte response to cytomegalovirus after human allogenic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood* 1991, 78, 1373–1380

37 Gilbert, M.J., Riddell, S.R., Li, C.-R. and Greenberg, P.D. Selective interference with class I major histocompatibility complex presentation of the major immediate–early protein following infection with human cytomegalovirus. *J. Virol.* 1993, 67, 3461–3469

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Induction of human cytomegalovirus (HCMV)-glycoprotein B (gB)-specific neutralizing antibody and phosphoprotein 65 (pp65)-specific cytotoxic T lymphocyte responses by naked DNA immunization

Valeria Endresz[a,b], Laszlo Kari[a], Klara Berencsi[a], Csaba Kari[a], Zsofia Gyulai[a], Csaba Jeney[a,1], Steve Pincus[c], Ulrich Rodeck[a], Claude Méric,[d], Stanley A. Plotkin[e], Eva Gönczöl[a,b,*]

[a]*The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA*
[b]*Department of Microbiology, A. Szent-Gyorgyi Medical University, Szeged, Hungary*
[c]*Virogenetics Corporation, Rensselaer Technology Park, Troy, NY 12180, USA*
[d]*Pasteur Mérieux Connaught, 1541, Avenue Marcel-Merieux, 69280 Marcy-l'Etoile, France*
[e]*Pasteur Mérieux Connaught, 3, Avenue Pasteur, B.P. 10-92430 Marnes-La-Coquette, France*

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Abstract

Plasmids expressing the human cytomegalovirus (HCMV) glycoprotein B (gB) (UL55) or phosphoprotein 65 (pp65) (UL83) were constructed and evaluated for their ability to induce immune responses in mice. The full-length gB as well as a truncated form expressing amino acids 1–680 of gB, and lacking the fragment encoding amino acids 681–907 including the transmembrane domain of gB (gB680) were evaluated. Immunization of mice with plasmids coding for gB or gB680 induced ELISA and neutralizing antibodies, with the highest titres in mice immunized with the gB680 plasmid. Mice immunized with the gB plasmid predominantly produced IgG2a gB-specific antibody, while the gB680 plasmid raised mostly IgG1 anti-gB antibody. Mice immunized with the pp65 plasmid developed pp65-specific cytotoxic T lymphocytes (CTL) and ELISA antibodies. Immunization with a mixture of both gB and pp65 plasmids raised antibodies to both proteins and pp65-specific CTL, indicating a lack of interference between these two plasmids. These results suggest that DNA immunization is a useful approach for vaccination against HCMV disease.

Keywords: HCMV; Antibody; CTL; DNA immunization

1. Introduction

Induction of specific cellular and humoral immune responses in experimental animals inoculated with plasmid vectors expressing viral proteins has been amply documented. Such studies have led to efforts to use naked DNA as vaccines against several viral diseases, including influenza [1–7], AIDS [8–10], rabies [11], hepatitis B [12–15] and hepatitis C [16], lymphocytic choriomeningitis [17–19], and herpes simplex virus- and murine cytomegalovirus (MCMV)-induced diseases [20–24].

Infection with cytomegalovirus (CMV) in humans is common and usually asymptomatic; however, the incidence and spectrum of disease in newborns and immunocompromised hosts establishes this virus as an important human pathogen [25]. CMV has also been suggested to be an important co-factor in the develop-

* Corresponding author. Fax: +1 215 898 3868; E-mail: gonczol@wista.wistar.upenn.edu.
[1] Present address: Department of Microbiology, Semmelweis Medical University, Budapest, Hungary.

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ment of atherosclerosis [26,27] and restenosis after angioplastic surgery [28,29].

Among the estimated 200 proteins encoded by the human cytomegalovirus (HCMV) genome [30], glycoprotein B (gB) [31–33] and phosphoprotein 65 (pp65) [34–37] have been implicated as principal targets of virus neutralizing antibody and virus-specific CTL responses, respectively. Work in our laboratory aimed at developing a recombinant vaccine to HCMV has shown that the gB protein when expressed by adenovirus [38], vaccinia virus [39], or canarypox (ALVAC) virus [40] vectors induces neutralizing antibodies in experimental animals, lymphocyte proliferation upon in vitro antigen stimulation [41], and CTL responses in mice of H-2k haplotype [42]. In this study, we constructed plasmids containing the full-length or truncated genes of HCMV-gB or the pp65 gene, respectively. gB is the major immunogenic protein of HCMV that induces neutralizing antibodies [31–33,39], and the 1-680 amino acid fragment of gB contains most of the neutralizing epitopes but lacks the transmembrane domain, resulting in the secretion of gB from cells [47,48]. Thus, these plasmids are suitable for evaluating whether the secreted form or the membrane-bound form of gB induces stronger NA responses. pp65 is the most prominent target of CTL responses after natural infection [34–37]. We report here 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. Mice immunized with the plasmid encoding gB produced predominantly IgG2a gB-specific antibody, while those immunized with the gB680 plasmid developed a greater antibody response dominated by IgG1 gB-specific antibody.

2. Materials and methods

2.1. Expression plasmids carrying HCMV gB (UL55) and pp65 (UL83) genes

2.1.1. pK-gB, pΔRC-gB and pΔRC-gB680 plasmids

The gB gene, derived from the plasmid pAd-gB [38], was subcloned into pUC-8, obtained as a *Hin*dIII–*Bam*HI fragment, and inserted into the pΔRC vector digested with *Hin*dIII and *Bam*HI (pΔRC-gB). pΔRC-gB680 was derived from pΔRC-gB by digestion with *Xho*I and removal of the C-terminal part of the gB gene. The pΔRC vector contains the CMV-IE promoter; it was derived from pRC/CMV (Invitrogen) by deleting the *Pvu*II 1290–*Pvu*II 3557 fragment to obtain more unique restriction sites. The full-length gB gene was also inserted to a plasmid expressing gB under the control of a tetracycline-regulatable promoter. This plasmid utilizes the tetracycline-regulatable system obtained from plasmids pUHD10-3 and pUHD15-1 (kindly provided by Dr H. Bujard, Heidelberg, Germany; described in Ref. [43]). The resulting plasmid, pK-gB contains: the tetracycline-regulatable promoter (seven tetracycline operators (Tet°) and the HCMV minimal promoter), HCMV-gB, SV40 poly A signal, HCMV-IE promoter/enhancer, tTA gene (encoding a fusion protein consisting of the tetracycline repressor from *Escherichia coli* and herpes simplex virus protein-16), and the SV40 poly A signal. The fusion protein is a powerful transactivator of the 7 Tet°-HCMV minimal promoter; however, in the presence of tetracycline, the transactivation is switched off, since tetracycline prevents the attachment of the tetracycline repressor to the tetracycline operator sequences. pK-gB expresses gB in a tetracycline-regulatable fashion: tetracycline (1 $\mu g\,ml^{-1}$) reduces gB expression, however, in the absence of tetracycline the tetracycline-regulatable promoter is fully functional and gB is expressed.

2.1.2. pΔRC-pp65 plasmid

First, the pp65 gene was cloned into a pUC-8 based plasmid. The HCMV pp65 gene was PCR-amplified using genomic DNA as template (Towne strain), oligonucleotides pp651 (5′GATTATCGCGATATCCGTTA AGTTTGTATCGTAATGGCATCCGTACTGGGT CCCATTTCGGG-3′) and pp651R (5′-GCATAGG TACCGGATCCATAAAAATCAACCTCGGTGCT TTTTGGGCG-3′), and Taq I polymerase (Perkin Elmer Cetus). The 1.6 kb product was digested with *Nru*I and *Bam*HI (sites present at the 5′ end of oligonucleotides pp651 and pp651R, respectively) and cloned into *Nru*I/*Bam*HI-digested SPHA-H6 (a pUC-8-based plasmid containing an irrelevant gene flanked by *Nru*I and *Bam*HI sites), generating plasmid CMV65.1 in which the first 30 bp of the pp65 gene were missing.

To derive a plasmid containing the entire pp65 gene, oligonucleotides RNApp65I (5′-TAGTTCGGATCCC CGCTCAGTCGCCTACA-3′) and pp65R4 (5′-ATC AAGGGATCCATCGAAAAAGAAGAGCG-3′) were used in PCR with genomic DNA. The resulting 1-kb fragment was digested with *Bam*HI (*Bam*HI sites present at the 5′ ends of both oligonucleotides) and cloned into *Bam*HI-digested IBI24 (International Biotechnologies), generating plasmid pp65.7. Plasmid pp65.7 was used in PCR with oligonucleotides pp651B (5′-GATTATCGCGATATCCGTTAAGTTTGTATC GTAATGGAGTCGCGCGGTCGCCGTTGTCCCG-3′) and pp65*Bst*XI (5′-ACCTGCATCTTGGTTGC-3′) to generate a 0.5-kb fragment. This fragment was digested with *Nru*I and *Bst*XI (sites at the 5′ ends of oligonucleotides pp651B and pp65*Bst*XI, respectively) and ligated to a 4.8-kb *Nru*I/*Bst*XI fragment of CMV65.1, generating plasmid pCMV65.2. The 1696-

bp pp65 gene was obtained from plasmid pCMV65.2 by *Nru*I–*Bam*HI digestion. The pΔRC-CMV vector was blunt-ended with Klenow polymerase, digested with *Bam*HI, and the pp65 gene was inserted.

Transient transfections of human 293 cells (ATCC) were performed using purified plasmid preparations (1.5 μg/3 × 10^5 cells), and Lipofectamine (BRL, Gaithersburg, MD). Two days after transfection, cells expressed the HCMV proteins as detected by immunofluorescence assay using gB-specific monoclonal antibody (Advanced Biotechnologies, Columbia, MD) and pp65-specific monoclonal antibody (Virostat, Portland, ME).

2.2. Preparation and purification of plasmids

Plasmids were transfected into *Escherichia coli* strain DH5α and stored at −70°C in medium and 10% glycerol solution. Bacteria were grown in LB broth supplemented with ampicillin. Large-scale purification was conducted using Quiagen Giga kits (Quiagen, Santa Clarita, CA) according to the manufacturer's protocol. Purity and concentration of DNA were determined by UV spectrophotometry. Purified plasmid DNA was aliquoted and stored at −70°C.

2.3. Immunization procedure

Seven- to ten-week-old female BALB/c mice were used in all immunizations. DNA was inoculated intramuscularly into the quadriceps at doses of 50–80 μg/mouse at times indicated in the specific experiments (see Results). In co-immunization experiments groups of mice were inoculated with either a mixture of both pK-gB and pΔRC-pp65 plasmids (80 μg of each DNA/mouse, 40 μg of each DNA/leg, 160 μg DNA/mouse), or each plasmid inoculated into two different legs (80 μg DNA of each plasmid/mouse, a total of 160 μg DNA/mouse inoculated in left and right legs). Booster injections were given 4 and 8 weeks later.

2.4. Microneutralization assay

HCMV neutralizing activity of sera was tested in a microneutralization assay [44]. Neutralizing titres higher than 1:8 were considered positive.

2.5. Cytotoxic T lymphocyte assay

The pp65-specific CTL assay was performed as previously described [42]. Briefly, spleen cells of immunized mice were restimulated in vitro with autologous spleen cells infected with vaccinia virus recombinant expressing HCMV-pp65 [42] (VacWR-pp65) or wild-type vaccinia (VacWR) for 5 days in 24-well plates (m.o.i = 0.2–0.5; effector:stimulator ratio, 2:1). Cytolytic activity of non-adherent spleen cells was tested in a 4-h ^{51}Cr-release assay. Target cells (P815 MHC class I-matched, MC57 MHC class I-mismatched) were infected with VacWR-pp65 or VacWR (m.o.i = 4–8). Percentage of specific ^{51}Cr-release was calculated as [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release) × 100].

2.6. ELISA assay

A preparation of gB protein purified by immunoaffinity column chromatography was used as coating antigen in assays to detect gB-specific serum antibody responses. Optical density values (A) higher than mean A +2 S.D. of pre-immune sera or A-values greater than or equal to 0.05 were considered positive, whichever was higher. For pp65-specific antibodies, lysates of 293 cells transiently transfected with pΔRC-pp65 were used as coating antigen, and lysates of untransfected 293 cells served as control antigen. For each serum dilution optical density values obtained in control antigen-coated wells were subtracted from those obtained in pp65 antigen-coated wells and were considered positive if they were greater than or equal to 0.05. Plates were blocked with PBS containing Tween 20 (0.05%, v/v) and skim milk (2.5% w/v) for 1 h. Serum samples and peroxidase-conjugated anti-mouse antibodies were diluted in blocking buffer and incubated sequentially on the plates. For colour development, 0.1 M citrate buffer (pH 4.5) containing hydrogen peroxide (0.012%, v/v) and O-phenylenediamine (1 mg/ml) was added for 15 min, at which point the reaction was stopped with 1 M sulfuric acid. Plates were read spectrophotometrically at 490 nm.

2.7. Isotype assay

Peroxidase-conjugated rabbit anti-mouse IgG3 and rat monoclonal anti-mouse IgG1, IgG2a, and IgG2b (Zymed, San Francisco, CA) were used to detect specific immunoglobulin isotypes. Each of the secondary antibodies was determined to be highly specific for the appropriate isotype and quantitatively similar in reactivity in the ELISA, allowing quantitative comparisons between the levels of each isotype. For determination of antigen-specific isotype content of sera, a standard curve was prepared for each isotype for each ELISA using purified mouse IgG1, IgG2a, or IgG2b (Cappel, Aurora, OH) as antigens. Serum titres were converted to antibody concentration by comparison with a standard, calculated from two points of the linear portion of the curve.

3. Results

3.1. pK-gB and pΔRC-pp65 induce insert-specific antibody and pp65-specific CTL responses in mice

Groups of 10 BALB/c mice were inoculated with pK-gB or pΔRC-pp65 (80 μg/mouse) and then boosted with the same amount of plasmid 4 and 8 weeks later. In mice immunized with pK-gB, HCMV-gB-specific ELISA antibodies were detected in 9 of 10 mice at 8 weeks and in all mice tested at 13 and 21 weeks (Fig. 1). Antibodies were still present in 4 of 5 mice tested at 31 weeks (Fig. 1). HCMV neutralizing antibodies were detected in 4 of 10 mice with titres ranging between 1:12 and 1:48 (data not shown). All mice immunized with pΔRC-pp65 responded with pp65-specific ELISA antibodies after the first booster (Fig. 2). Six mice were sacrificed at week 13 for CTL analysis. Three responded with pp65 specific lysis of target cells (Fig. 3). All four remaining mice had significant ELISA antibodies at week 13 (Fig. 2). Parental vector plasmids did not induce gB or pp65-specific ELISA antibodies. In additional experiments, 5 of 9 mice immunized with pΔRC-pp65 showed strong pp65-specific CTL responses (data not shown).

3.2. Co-immunization with a mixture of pK-gB and pΔRC-pp65 induces antibody and pp65-specific CTL responses

To determine whether the injection of a mixture of the two plasmids pK-gB and pΔRC-pp65 had an effect on the immune response towards each encoded antigen, mice were immunized either with a mixture of both plasmids or with each plasmid at different sites.

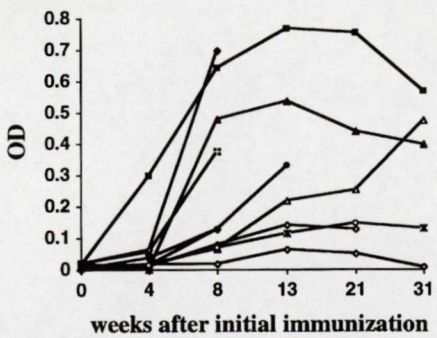


Fig. 1. pK-gB induces gB-specific antibody in mice. Mice were immunized with pK-gB and boosted at weeks 4 and 8. Serum samples were analyzed at the indicated times by gB-specific ELISA. Optical density values obtained with 1:40 dilution of serum are shown. Each curve represents results of individual mice. Some mice died during the course of the study as a consequence of anaesthesia and/or bleeding.

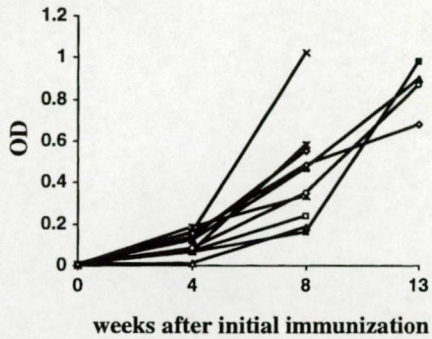


Fig. 2. pΔRC-pp65 elicits pp65-specific antibody responses. Mice were immunized with 80 μg pΔRC-pp65 and boosted at weeks 4 and 8. Serum samples were analysed at the indicated times by pp65-specific ELISA. pp65-specific optical density values obtained with 1:40 dilution of serum are shown. Each curve represents results of individual mice.

They were boosted 1 and 2 months later, and serum samples collected at different times after the initial immunization were assayed for both gB and pp65 antibodies. All ten mice inoculated with the plasmid mixture developed both gB (Fig. 4A) and pp65 (Fig. 4B) specific antibodies 8 or 13 weeks after the first inoculation and in those mice where serum samples were assayed, antibodies were detected

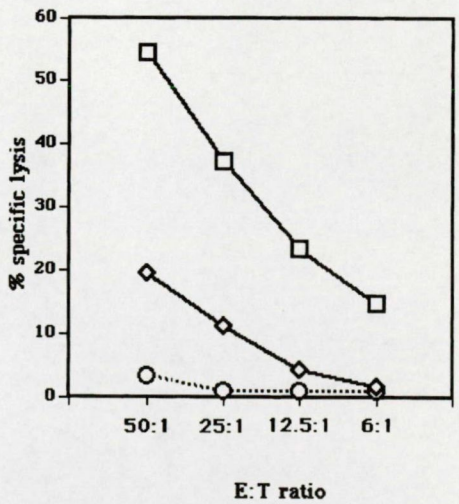


Fig. 3. pp65-specific CTL responses in BALB/c mice immunized with pΔRC-pp65. Mice were immunized as described in Fig. 2 and sacrificed 1 month after booster. Spleen cell cultures were restimulated with VacWR-pp65-infected autologous spleen cells for 5 days. Specific lysis was tested on WT-Vac- or VacWR-pp65-infected P-815 (MHC-matched) and VacWR-pp65-infected MC57 (MHC-mismatched) target cells. The figure shows the result obtained with one of the three CTL-responder mice. □, P-815 VacWR-pp65-infected; ◇, P-815 WT-Vac-infected; ○, MC57 VacWR-pp65-infected.

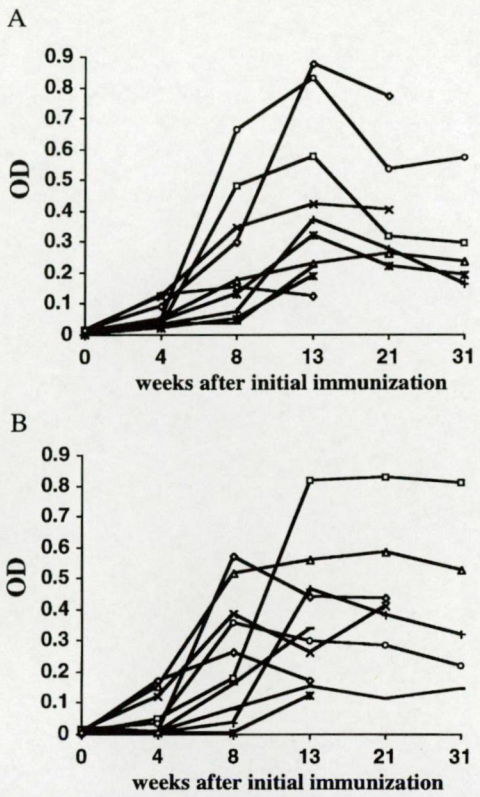


Fig. 4. Kinetics of gB and pp65-specific antibody responses of mice immunized with a mixture of pK-gB and pΔRC-pp65. Mice were immunized with a mixture of 80 μg pK-gB and 80 μg pΔRC-pp65 and boosted at weeks 4 and 8. Serum samples were analysed at the indicated times by gB and pp65-specific ELISA. (A) and (B) show gB-specific and pp65-specific optical density values, respectively, obtained with 1:40 dilution of individual serum samples. Some mice died during the course of the study as a consequence of anaesthesia and/or bleeding.

throughout the 31-week observation period. The time course of the gB- and pp65-specific ELISA antibody response in the group immunized with the mixture was not significantly different from that of the group immunized with the two plasmids at separate sites (data not shown).

In another experiment, similar gB- and pp65-specific antibody responses were observed. A pp65-specific CTL response was detected in 4 of 5 mice immunized with a mixture of both plasmids, while a pp65-specific CTL response was obtained in 4 of 6 mice that received the two plasmids at different sites (data not shown).

These results establish that the injection at the same site of a mixture of plasmids coding for pp65 and gB does not affect the specific immune response directed towards each antigen.

3.3. Immunization with the secreted form of gB (pΔRC-gB680) induces a greater gB-specific ELISA and neutralizing antibody response than immunization with the natural membrane-anchored form of gB (pΔRC-gB)

To determine whether a secreted form of gB would induce a greater level of antibody response than the native membrane-anchored form of the protein, two kinds of gB-expressing plasmids were constructed: pΔRC-gB expresses the full-length protein and pΔRC-gB680 expresses gB lacking the transmembrane domain of the protein. Each plasmid was injected at 0 and 8 weeks in different groups of mice and the ELISA response was assessed at 12 weeks. The mice of the pΔRC-gB680 group responded considerably better than the mice of the pΔRC-gB group. In the pΔRC-gB680 group, 9 of 10 mice responded with a mean optical density value of 2.833 ± 0.194, while 6 of 10 mice in the pΔRC-gB group responded with a mean optical density value of only 0.436 ± 0.470 at a dilution of 1:40. The difference is statistically significant ($p < 0.01$) by Student's t-test. As shown in Fig. 5, there is also a difference between the two groups when the neutralizing antibody responses are compared. In the pΔRC-gB680-immunized group, 10 of 10 mice had neutralizing antibodies with titres ranging from 1:12 to 1:192, whereas only 4 of 10 mice immunized with pΔRC-gB responded with titres of 1:12 to 1:48.

3.4. Different gB-specific IgG isotypes are generated by the membrane-anchored and the secreted form of gB

Because the way an antigen is presented to the immune system can affect the T-helper cell type and ratio of antibody isotypes that are induced [45, 46], we tested whether antibody isotypes might be differentially

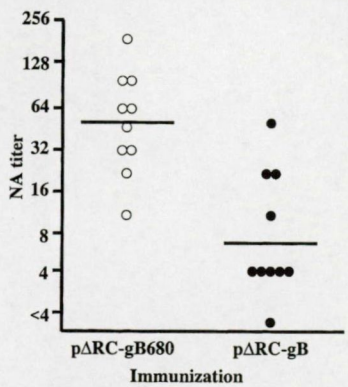
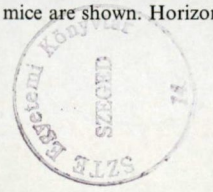


Fig. 5. Higher HCMV neutralizing antibody titres are elicited by pΔRC-gB680 than by pΔRC-gB. Mice were immunized with 50 μg pΔRC-gB or pΔRC-gB680, and a corresponding booster was given 8 weeks later. Sera were obtained 8 weeks after the booster and tested for HCMV neutralizing antibodies. Neutralizing antibody titres of individual mice are shown. Horizontal bars show geometric means.



affected by the immunization with plasmids coding for the membrane-bound form of gB versus the secreted form. Two groups of mice received two immunizations of pΔRC-gB or pΔRC-gB680 8 weeks apart and individual sera obtained 4 weeks after the booster were analysed for gB antibody in IgG isotype-specific ELISA assays. Both forms of the protein generated IgG1 and/or IgG2a gB-specific antibodies (Table 1) while other antibody isotypes, including IgG2b and IgG3, were either undetectable or present in only marginal amounts. Both IgG1 and IgG2a antibodies were formed after pΔRC-gB immunization, although IgG2a antibodies were much more abundant; the ratio of IgG1/IgG2a was less than 1 in all but one mouse. Mice in the pΔRC-gB80-immunized group also developed both IgG1 and IgG2a antibody, but IgG2a was less abundant than IgG1 (except in one mouse), and thus IgG1:IgG2a ratios were greater than 1 in these mice (Table 1).

4. Discussion

The pΔRC-pp65 and the gB plasmids expressing the full-length gB or the 1–680 amino acid fragment of the gB polypeptide proved to be good immunogens. However, the secreted form of gB induced significantly higher ELISA and neutralizing titres than did the membrane-bound full-length gB (Fig. 5). It remains unclear why pΔRC-gB680 is a better immunogen; one possibility is that the mechanism of antigen presentation is different for the membrane-bound and secreted gB. The membrane-bound gB may be presented by transfected myocytes and/or professional antigen-presenting cells (APC) that become transfected at the site of inoculation, while a secreted protein can be taken up and presented by non-transfected resident or recruited APCs [11,49]. Our findings are that the membrane-bound form of gB produces an IgG2a-dominant response, whereas similar inoculations with the secreted gB-expressing plasmid generates a predominantly IgG1 response suggesting a different antigen presentation mechanism. Several studies have demonstrated IgG2a-dominant immune responses after intramuscular DNA immunization with membrane-bound protein or intracellularly localized protein expressing plasmids [11,22,50]. Feltquate et al. [51] compared the IgG isotype pattern after immunization with DNA expressing the membrane-bound or secreted form of influenza haemagglutinin (HA); predominant IgG2a induction using the whole protein and IgG1 using the secreted form of HA was demonstrated. The biological significance of the differential isotype pattern induced by the membrane-bound and secreted gB is not known. In sera from healthy blood donors, IgG1 was the most frequently detected gB-specific subclass [52], and a range of human gB-specific neutralizing monoclonal antibodies were found to belong to the IgG1 isotype [53]. The human IgG1 subclass is the functional analogue of murine IgG2a, and in agreement with the correlation between neutralizing activity and IgG1 subclass in humans, in mice immunized with gB and QS21 adjuvant, a significant correlation was found between the levels of virus-neutralizing antibodies and IgG2a anti-gB antibodies [54]. On the other hand, immunization with gB given together either with aluminum hydroxide or Freund's adjuvant resulted in the production of only IgG1 antibodies, and these antibodies also neutralized the virus [45,54]. Other studies also showed that neutralizing CMV-specific mouse monoclonal antibodies may belong to either the IgG1, IgG2a or IgG2b isotypes [55–57]. Moreover, a synergistic effect was described between two monoclonal antibodies in a virus neutralization assay, where one monoclonal antibody had a much greater neutralizing activity in the presence of a non-neutralizing IgG1 antibody [55]. Further studies are needed to demonstrate the possible role of gB-specific IgG subclasses in vivo.

We did not test gB-specific CTL responses since the major function of the gB protein is considered to be the induction of neutralizing antibodies. We also showed previously that BALB/c mice do not respond with gB-specific CTL after immunization with an adenovirus-gB recombinant, although CBA mice showed a strong CTL response [42]. However, in view of recent studies on hepatitis B virus surface antigen, showing that DNA immunization can induce insert-specific CTL in mice previously considered non-

Table 1
Isotypes of anti-gB Ab generated by immunization with plasmids expressing the membrane-bound or the secreted form of gB

Immunization	Mouse #	Anti-gB Ab[a]	
		IgG1	IgG2a
pΔRC-gB	1	20	120
	2	< 10	400
	3	25	67
	4	45	30
	5	< 10	82
	6	50	140
pΔRC-gB680	1	928	98
	2	116	204
	3	191	89
	4	114	66
	5	212	74
	6	306	90
	7	611	17

[a]Mice were inoculated with 50 μg of pΔRC-gB or pΔRC-gB680, and boosted 8 weeks later under the same conditions. Sera obtained after booster were assayed for isotype-specific anti-gB Ab. Values are given as μg Ab per 1 ml of serum.

responders [15], we cannot exclude the possibility of gB-specific CTL induction in BALB/c mice. We did test for pp65-specific CTL responses, since the pp65 protein is a predominant protein for CTL induction in natural infections [34–37]. Indeed, pp65-specific ELISA antibodies were detected in all mice, and pp65-specific CTL responses were found in 16 of the 26 mice tested. It remains unclear why some of the mice failed to demonstrate pp65-specific CTL activity. Fuller and Haynes [8] also reported variable CTL responses after immunization of mice with a DNA-based HIV glycoprotein 120 vaccine.

Pande et al. [58] reported the induction of pp65-specific ELISA antibodies by DNA immunization using two plasmid expression vectors, in which the inserted gene was expressed under the control of the human β-actin promoter or the HCMV IE promoter along with intron A. However, neither plasmid construct induced ELISA antibodies in more than 60% of the inoculated mice after two inoculations [58], whereas nearly 100% of mice in our study responded with pp65-specific ELISA antibodies. The difference in the ELISA antibody responses in these studies might reside in the use of different vector plasmids, inoculation protocols and antibody detection systems. Our results also demonstrate that DNA immunization induces CTL specific for pp65.

Although specific immunity following vaccination can last for years or even a lifetime, some vaccines induce only short-term protection. The physical properties of the antigen, its in vivo persistence, route of inoculation, dose and frequency of reinoculation all may influence the duration of immune response. In our study, pp65- and gB-specific antibody responses were detectable by ELISA in mice for the duration of the observation period, i.e., for nearly 8 months after the first inoculation with pΔRC-pp65 or pK-gB, respectively. Long-lasting antibody responses have been demonstrated in mice immunized with DNA expressing the influenza virus haemagglutinin and nucleoprotein [3, 59], the hepatitis B virus surface antigen [14] and rabies virus glycoprotein G [11]. In contrast, only transient antibody responses were observed in macaques after repeated inoculation with a plasmid DNA expressing a simian immunodeficiency virus protein [10] and in mice immunized with DNA expressing the circumsporozoite protein of malaria [60].

pK-gB inoculated in combination with pΔRC-pp65, given as a mixture or separately, induced gB- and pp65-specific ELISA antibodies in nearly all mice. Four of 5 mice immunized with the mixture of two plasmids showed pp65-specific CTL. The presence of both gB- and pp65-specific ELISA antibodies and pp65-specific CTL in the mice inoculated with the mixture of the two plasmids indicates that gB and pp65 do not mutually block antigen presentation or B and T cell stimulation when expressed in the same cells or in close vicinity.

Our results show that DNA immunization can be carried out successfully with plasmid DNA expressing the HCMV gB and pp65 proteins and that the secreted form of gB is more immunogenic than the membrane-bound full-length gB. A combination of two plasmids expressing the gB and pp65 proteins or a single chimeric plasmid would induce both cellular and humoral immune responses important in protection against CMV disease.

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References

[1] Donnelly JJ, Friedman A, Martinez DL et al. Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. Nature Med. 1995;1:583–7.
[2] Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. USA 1993;90:11478–82.
[3] Raz E, Carson DA, Parker SE et al. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity- to viruses. Proc. Natl. Acad. Sci. USA 1994;91:9519–23.
[4] Robinson HL, Hunt LA, Webster RG. Protection against a lethal influenza virus challenge by immunization with a hemagglutinin-expressing plasmid DNA. Vaccine 1993;11:957–60.
[5] Robinson, HL, Feltquate, DM, Morin, MJ, 1995. DNA vaccines: a new approach to immunization. In: Brown F, Chanock R, Ginsberg H (Eds.), Vaccines 1995. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 69–75.
[6] Ulmer JB, Donnelly JJ, Parker SE et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993;259:1745–9.
[7] Webster RG, Fynan EF, Santoro JC, Robinson H. Protection of ferrets against influenza challenge with a DNA vaccine to the hemagglutinin. Vaccine 1994;12:1495–504.
[8] Fuller DH, Haynes JR. A qualitative progression in HIV type I glycoprotein 120-specific cytotoxic cellular and humoral immune response in mice receiving a DNA based glycoprotein 120 vaccine. AIDS Res. Human Retroviruses 1994;10:1433–41.
[9] Lu S, Santoro JC, Fuller DH, Haynes JR, Robinson HL. Use of DNAs expressing HIV-1 Env and noninfectious HIV-1 particles to raise antibody responses in mice. Virology 1995;209:147–54.
[10] Lu S, Arthos J, Montefiori DC et al. Simian immunodeficiency virus DNA vaccine trial in macaques. Journal of Virology 1996;70:3978–91.
[11] Xiang ZQ, Spitalnik S, Tran M, Wunner WH, Cheng J, Ertl HCJ. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology 1994;199:132–40.

[12] Davis HL, Schirmbeck R, Reimann J, Whalen RG. DNA-mediated immunization in mice induces a potent MHC class I-restricted cytotoxic T lymphocyte response to the hepatitis B envelope protein. Human Gene Therapy 1995;6:1446–56.
[13] Kuhöber A, Pudollek H-P, Reifenberg K et al. DNA immunization induced antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2^b mice. J. Immunol. 1996;156:3687–95.
[14] Michel M-L, Davis HL, Schleef M, Mancini M, Tiollais P, Whalen RG. DNA-mediated immunization to the hepatitis B surface antigen in mice: aspects of the humoral response mimic hepatitis B viral infection in humans. Proc. Natl. Acad. Sci. USA 1995;92:5307–11.
[15] Schirmbeck R, Böhm W, Ando K, Chisari FV, Reimann J. Nucleic acid vaccination primes hepatitis B virus surface antigen-specific cytotoxic T lymphocytes in nonresponder mice. J. Virol. 1995;69:5929–34.
[16] Major ME, Vitvitski L, Mink MA et al. DNA-based immunization with chimeric vectors for the induction of immune responses against the hepatitis C virus nucleocapsid. J. Virol. 1995;69:5798–805.
[17] Martins LP, Lau LL, Asano MS, Ahmed R. DNA vaccination against persistent viral infection. J. Virol. 1995;69:2574–82.
[18] Yokoyama M, Zhang J, Whitton L. DNA immunization confers protection against lethal lymphocytic choriomeningitis virus infection. J. Virol. 1995;69:2684–8.
[19] Zarozinski CC, Fynan EF, Selin LK, Robinson HL, Welsy RM. Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle bombardment with DNA encoding an internal virion protein. J. Immunol. 1995;154:4010–7.
[20] Bourne N, Stanberry LR, Bernstein DI, Lew D. DNA immunization against experimental genital herpes simplex virus infection. J. Infect. Dis. 1996;173:800–7.
[21] Cox GJM, Zamb TJ, Babiuk LA. Bovine herpesvirus 1: immune responses in mice and cattle injected with plasmid DNA. J. Virol. 1993;67:5664–7.
[22] Manickan E, Rouse RJD, Yu Z, Wire WS, Rouse BT. Genetic immunization against herpes simplex virus. Protection is mediated by CD4$^+$ T lymphocytes. J. Immunol. 1995;155:259–65.
[23] McClements WL, Armstrong ME, Keys RD, Liu MA. Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of herpes simplex virus-2 disease. Proc. Natl. Acad. Sci. USA 1996;93:11414–20.
[24] González Armas JC, Morello CS, Cranmer LD, Spector DH. DNA immunization confers protection against murine cytomegalovirus infection. J. Virol. 1996;70:7921–8.
[25] Britt WJ, Alford CA, 1996. Cytomegalovirus. In: Fields, B., Knipe, N., Howley, D.M.P.M. et al. (Eds.), Virology. Lippincott-Raven, Philadelphia, PA, pp. 2493–2523.
[26] Melnick JL, Adam E, DeBakey ME. Cytomegalovirus and atherosclerosis. BioEssays 1995;17:899–903.
[27] Berencsi K, Endresz V, Klurfeld D, Kari L, Kritchevsky D, Gönczöl E, 1997. Early atherosclerotic lesions in the aortic wall of the mouse induced by cytomegalovirus infection. Cell Adhesion Commun., in press.
[28] Speir E, Modali R, Huang E-S et al. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 1994;265:391–4.
[29] Zhou YF, Leon MB, Waclawiw MA et al. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. N. Engl. J. Med. 1996;335:624–30.
[30] Chee MS, Bankier AT, Beck S et al. Analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. Curr. Topics Microbiol. Immunol. 1990;154:125–69.
[31] Britt WJ, Vugler L, Butfiloski EJ, Stephens EB. Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of human neutralizing antibody response. J. Virol. 1990;64:1079–85.
[32] Gönczöl E, Ianocone J, Ho W-Z, Starr S, Meigneir B, Plotkin SA. Isolated gA/gB glycoprotein complex of human cytomegalovirus envelope induces humoral and cellular immune responses in human volunteers. Vaccine 1990;8:130–6.
[33] Marshall GS, Rabalais GP, Stout GG, Waldeyer SL. Antibodies to recombinant-derived glycoprotein B after natural human cytomegalovirus infection correlate with neutralizing activity. J. Infect. Dis. 1992;165:381–4.
[34] McLaughlin-Taylor E, Pande H, Forman SJ et al. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8$^+$ virus-specific cytotoxic T lymphocytes. J. Med. Virol. 1994;43:103–10.
[35] Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood 1991;78:1373–80.
[36] Riddell SR, Rabin M, Geballe AP, Britt WJ, Greenberg PD. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. J. Immunol. 1991;146:2795–804.
[37] Wills MR, Carmichael AJ, Mynard K et al. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J. Virol. 1996;70:7569–79.
[38] Marshall GS, Ricciardi RP, Rando RF et al. An adenovirus recombinant that expresses the human cytomegalovirus major envelope glycoprotein and induces neutralizing antibodies. J. Infect. Dis. 1990;162:1177–81.
[39] Gönczöl E, deTaisne C, Hirka G et al. High expression of human cytomegalovirus (HCMV)-gB protein in cells infected with a vaccinia-gB recombinant; the importance of the gB protein in HCMV-immunity. Vaccine 1991;9:631–7.
[40] Gönczöl E, Berencsi K, Pincus S et al. Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate. Vaccine 1995;13:1080–5.
[41] Berencsi K, Gönczöl E, Valyi-Nagy T, et al., 1991. Recombinant human cytomegalovirus (HCMV)-gB glycoprotein induces gB-specific cell-mediated immunity in mice. In: Landini MP (Ed.), Progress in Cytomegalovirus Research, Excerpta Medica Proceedings of the 3rd International Cytomegalovirus Workshop, pp. 191–194.
[42] Berencsi K, Rando R, deTaisne C, Paoletti E, Plotkin SA, Gönczöl E. Murine cytotoxic T cell response specific for human cytomegalovirus glycoprotein B (gB) induced by adenovirus and vaccinia virus recombinants expressing gB. J. Gen. Virol. 1993;74:2507–12.
[43] Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA 1992;89:5547–51.
[44] Gönczöl E, Furlini G, Ianacone J, Plotkin SA. A rapid microneutralization assay for cytomegalovirus. J. Virol. Methods 1986;14:37–41.
[45] Gönczöl E, Berencsi K, Kauffman E et al. Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate: immune response elicited in a prime/boost protocol with the glycoprotein B subunit. Scand. J. Infect. Dis. (Suppl.) 1995;99:110–2.

[46] Hocart MJ, Mackenzie JS, Stewart GA. The IgG subclass responses to influenza virus haemagglutinin in the mouse: effect of route of inoculation. J. Gen. Virol. 1989;70:809–18.

[47] Banks T, Huo B, Kousoulas K, Spaete R, Pachl C, Pereira L. A major neutralizing domain maps within the carboxyl-terminal half of the cleaved cytomegalovirus B glycoprotein. J. Gen. Virol. 1989;70:979–85.

[48] Spaete RR, Gehrz RC, Landini MP. Human cytomegalovirus structural proteins. J. Gen. Virol. 1994;75:3287–308.

[49] Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu. Rev. Immunol. 1997;15:617–8.

[50] Raz E, Tighe H, Sato Y et al. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc. Natl. Acad. Sci. USA 1996;93:5141–5.

[51] Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J. Immunol. 1997;158:2278–84.

[52] Urban M, Winkler T, Landini MP, Britt W, Mach M. Epitope-specific distribution of IgG subclasses against antigenic domains on glycoproteins of human cytomegalovirus. J. Infect. Dis. 1994;169:83–90.

[53] Tomiyama T, Matsumoto Y-I, Sugano T, Fujinaga S, Masuho Y. Preparation of human monoclonal antibodies against a cytomegalovirus glycoprotein complex of 130 and 55 kDa. J. Immunol. Meth. 1990;131:249–55.

[54] Britt W, Fay J, Seals J, Kensil C. Formulation of an immunogenic human cytomegalovirus vaccine: responses in mice. J. Infect. Dis. 1995;171:18–25.

[55] Lussenhop NO, Goertz R, Wabuke-Bunoti M, Gehrz R, Kari B. Epitope analysis of human cytomegalovirus glycoprotein complexes using murine monoclonal antibodies. Virology 1988;164:362–72.

[56] Rasmussen L, Mullenax J, Nelson R, Merigan TC. Viral polypeptides detected by a complement-dependent neutralizing murine monoclonal antibody to human cytomegalovirus. J. Virol. 1985;55:274–80.

[57] Britt WJ. Neutralizing antibodies detect a disulfide-linked glycoprotein complex within the envelope of human cytomegalovirus. Virology 1984;135:369–78.

[58] Pande H, Campo K, Tanamachi B, Forman SJ, Zaia JA. Direct DNA immunization of mice with plasmid DNA encoding the tegument protein pp65 (ppUL83) of human cytomegalovirus induces high levels of circulating antibody to the encoded protein. Scand. J. Infect. Dis. 1995;99:117–20.

[59] Yankauckas MA, Morrow JE, Parker SE et al. Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmid DNA containing NP gene. DNA Cell Biol. 1993;12:771–6.

[60] Mor G, Klinman DM, Shapiro S et al. Complexity of the cytokine and antibody response elicited by immunizing mice with *Plasmodium yoelii* circumsporozoite protein plasmid DNA. J. Immunol. 1995;155:2039–46.

VI.

ÖSSZEFOGLALÓ

Új vakcinák lehetősége a jövő század első negyedében

Gönczöl Éva
Berencsi Klára
Endrész Valéria
Burián Katalin
Gyulai Zsófia
Kari László
Virók Dezső

POSSIBLE NEW VACCINATIONS IN THE FIRST QUARTER OF THE NEXT CENTURY

The WHO-directed vaccination campaign against smallpox in the late 1970s led to the eradication of the variola virus and made the anti-smallpox vaccination unnecessary. The currently available vaccines have also provided substantial protection against various infectious diseases. "Conventional" vaccines (containing attenuated live or inactivated pathogens or their immunogenic components), however, would not afford protection against many bacterium-, virus- or parasite-caused diseases, or their use would not meet the necessary safety requirements. The increasing knowledge in molecular biology relating to these pathogens and basic immunology, and also revolutionary progress in gene technology, have provided alternative possibilities for the development of vaccines against such pathogens. One of the alternatives is the production and use of bacterial or viral proteins engineered by expression vectors (bacterium, virus, yeast or plant), which would induce humoral immune responses in the immunised individuals. Another alternative is the use of recombinant vectors themselves (bacterium, virus or plasmid) for immunisation, which would induce not only humoral, but also cellular immune response against the inserted gene product. The most promising vectors are the virus vectors, which do not fully replicate, but express early genes in human cells, and the eukaryotic plasmid vectors (DNA immunisation). The application of molecular biology in preventive approaches, will soon lead to the development of a number of effective and safe vaccines against severe human and animal diseases.

Correspondence:
Éva Gönczöl, MD
Szent-Györgyi Albert
Medical University
Department of Microbiology
H-6720 Szeged, Dóm tér 10.

recombinant vaccines, DNA-immunisation, new vaccines

A himlőmegbetegedés elleni vakcinációs kampánnyal a WHO az 1970-es évek végén himlővírusmentessé tette a Földet, és a himlő elleni vakcinációt megszüntette. A jelenleg forgalomban lévő baktérium- és vírusvakcinák is jelentős védelmet biztosítanak számos fertőző betegséggel szemben. Sok vírus, baktérium és parazita okozta megbetegedéssel szemben azonban a konvencionális úton előállított vakcinák (élő attenuált vagy inaktivált mikroorganizmusok vagy azok immunogén alkotóelemei) nem biztosítanak védelmet, vagy alkalmazásuk nem biztonságos. Ezen patogének molekuláris biológiai ismerete és a géntechnológia fejlődése alternatív megoldásokat tesz lehetővé; ezek között említhetők az expressziós vektorokkal (vírusok, baktériumok, gombák, növények) termeltetett baktérium- vagy vírusfehérjék (a patogénnek a vektorba beültetett génproduktumai), amelyek humorális immunválaszt hoznak létre az immunizált egyénben. Az expressziós vektoroknak (baktériumok, vírusok és plazmidok) az immunizálásra való felhasználása azonban még hatásosabb, azaz mind celluláris, mind humorális immunválasz létrejön. Az immunizálásra potenciálisan használható vektorok között az emberi sejtekben nem replikálódó, de a korai géneket kifejező vírusvektorok és az expressziós eukaryotaplazmid-vektorok (DNS-vakcinák) ígéretesek. A fenti molekuláris biológiai technikák alkalmazásával a következő években több új hatásos és biztonságos vakcina kerül általános használatra.

Levelezési cím:
dr. Gönczöl Éva
Szent-Györgyi Albert
Orvostudományi Egyetem
Mikrobiológiai Intézet
6720 Szeged, Dóm tér 10.

rekombináns vakcinák, DNS-immunizálás, új vakcinák

Új cikksorozatunkban jeles szakemberek tárják az olvasók elé az orvoslás egyes szakterületeinek ezredvégi kihívásait.

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Az orvostudomány eddigi sikerei közé sorolhatjuk a vakcináció terén elért eredményeket. Ezek között talán legnagyobb a himlőbetegség és a himlővírus-fertőzés lehetőségének teljes kiiktatása, amelyet az Egészségügyi Világszervezet (World Health Organization, WHO) az 1970-es évek végén világraszóló oltási kampánnyal bonyolított le. A kampány után a WHO a himlőoltás megszüntetését javasolta, és az oltást 1980-ban világszerte elhagyták *(1)*. Ugyancsak sikert jelentett az 1950-es évek végén az elölt (Salk), majd az 1960-as évek elején az élő attenuált poliomyelitis-oltóanyagok (Sabin) kiterjedt alkalmazása, amelynek hatására gyakorlatilag nem fordul elő fertőzés a vad poliomyelitisvírussal. A WHO a himlővakcinációs kampány analógiája alapján poliomyelitiskampányt tervez a 2000-es évek elején, amelytől a világ poliomyelitismentességét reméli. Ezt követné a kanyaróvírus eradikációja, szintén az egész világra kiterjedő oltási kampány eredményeképpen, amelyhez élő attenuált kanyaróvírust tartalmazó oltóanyagot használnak majd. A fentieken túlmenően hazánkban a kötelező élő attenuált vírusvakcinák, mint a mumpsz és a rubeola, az élő attenuált bakteriális vakcina, mint a tuberkulózis (BCG), az inaktivált bakteriális vakcinák közül a szamárköhögés (*Bordetella pertussis*) -vakcina biztosítanak lényeges védelmet. Ezen konvencionális vakcinák tehát vagy a patogén inaktiválásával, azaz elölésével (elölt poliomyelitis, *B. pertussis*), vagy a patogén attenuálásával, azaz virulenciájának megszüntetésével, de ugyanakkor immunogenitásának megtartásával (élő attenuált poliomyelitis, kanyaró, mumpsz, rubeola, BCG) kerülnek előállításra *(2–4)*.

A patogén attenuálása rendszerint évekig tartó, szuboptimális körülmények között végzett, sorozatos továbboltással történt, és a vakcinációra alkalmas mikroorganizmusegyedeket úgy választották ki, hogy a továbboltott kultúrák virulenciáját és immunogenitását kísérleti állatok szervezetében mérték. Ilyen módon az attenuálás során véletlenszerű mutációk jöttek létre a patogén genomjában, amelyek a virulencia elvesztését és az immunogenitás megtartását eredményezték: a mutáns egyedeket in vivo teszteléssel választották ki. Jelenleg, a vírusok genomjának ismeretében, a patogenitásért felelős gének genetikai módosításával végzik az attenuálást.

A baktériumok protektív hatásért felelős komponenseinek – amelyek sok esetben a patogenitásért felelős komponensek is – felhasználása oltóanyagként szintén gyakorlatban van, természetesen a patogén hatásért felelős mechanizmus inaktiválása után. Ilyenek a diphtheria- és tetanusexotoxinokból készített toxoidok, amelyek a kötelező oltások közé tartoznak. Továbbá válogatott, nagy rizikójú csoportba tartozó emberek vakcinációja lehetséges a baktérium más alkotórészeinek használatával is, ilyenek a *Neisseria meningitidis*-, a *Haemophilus influenzae*- és a *Streptococcus pneumoniae*-poliszacharidavakcinák. A *H. influenzae* b (Hib)-poliszacharida-hordozó fehérjéhez (tetanustoxoid) kötve hatásosabb immunválaszt hoz létre (hosszabb ideig tartó B- és T-sejt-aktivációt is jelentő választ), és hazánkban 1999-től az általános használatra kijelölt vakcinák közé tartozik *(5)*. Járványveszély, endémiás területre utazás vagy feltételezetten megtörtént fertőzés esetén további vakcinák állnak rendelkezésünkre, ezek közé tartoznak a *Yersinia pestis*-, a *Vibrio cholerae*-baktérium-, a hepatitis A-, a sárgaláz-, a kullancsencephalitis- és a veszettségvírus-vakcinák. Élő attenuált varicellavakcinát állítottak elő japán kutatók az 1980-as években, amelyet 1987 óta Japánban és 1995 óta az Egyesült Államokban jó eredménnyel használnak. Hazánkban még csak a nagy rizikójú csoportba tartozó (leukaemiás) gyermekek vakcinációját engedélyezték, de remélhetőleg a varicellavakcina az általános vakcinációs programba is hamarosan bekerül *(2–5)*.

A molekuláris biológia eredményeit felhasználó,

- Bizonyos vírusoknál és intracellulárisan szaporodó baktériumoknál olyan mechanizmusok alakultak ki, amelyek segítségével a fertőzés kiváltotta immunválasz elől el tudnak menekülni, azaz az immunválasz nem tudja őket elpusztítani.
- A patogének másik csoportjával történt fertőzés vagy vakcináció kivált ugyan protektív immunválaszt, de a kórokozó különböző törzsei közötti antigénkülönbségek (például a rhinovirusoknál) vagy a patogén felszíni antigénjeinek gyakori változása (például az influenzavírus és a HIV esetében) miatt az egyén védtelen az új antigénstruktúrával rendelkező patogénnel szemben, tehát újrafertőződik.
- Vektor az a mikroorganizmus vagy plazmid, amely genetikai állományába egy másik mikroorganizmus valamilyen génjét klónozták, amely gént a vektor sajátjaként fejez ki és termel.
- A kanáripox rekombináns vírusok protektív immunválaszt váltanak ki különböző emlősfajokban.
- Az izomba vagy bőrbe inokulált DNS az általa kódolt fehérjét kifejezve immunogén, és immunválaszt vált ki.
- Ha ismert, hogy a vírus vagy baktérium melyik alkotóeleme váltja ki a protektív immunválaszt, ezen alkotórészt vakcinaként lehet használni.

dr. Gönczöl Éva
dr. Endrész Valéria
dr. Burián Katalin
dr. Gyulai Zsófia
dr. Virók Dezső
Szent-Györgyi Albert Orvostudományi Egyetem
Mikrobiológiai Intézet
dr. Berencsi Klára
dr. Kari László
The Wistar Institute
Philadelphia,
USA

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esetenként azt bővítő, modern vakcinológia első, a gyakorlatban is alkalmazást nyert terméke a hepatitis B felszíni antigént (HBsAg) tartalmazó rekombináns vakcina. A rekombináns HBsAg-vakcina kidolgozásakor a felszíni antigént kódoló gént élesztőgomba DNS-ébe klónozták, majd ezt a rekombináns élesztőgombát nagy mennyiségben elszaporították, és a tenyészkultúrából a HBsAg-t tisztítási eljárással állították elő *(6)*. A vakcina háromszori oltás után védő hatású. A hepatitis B-vírus-fertőzésnek fokozottan kitett egyének – mint például a vírushordozó anyák gyermekei, a vírust hordozó betegek hozzátartozói, vérkészítményeket rendszeresen kapó betegek, homoszexuálisok, egészségügyi munkát végzők – eddig is részesülhettek oltásban, de 1999-től a HBsAg az általános használatra alkalmazott vakcinák közé tartozik.

A vakcinációval megelőzhető betegségekénél azonban hosszabb listát lehet készíteni azokból a betegségekből, amelyeknek kialakulását nem tudjuk védőoltással megakadályozni. A teljesség igénye nélkül említjük a fertőző légúti betegségeket, amelyek minden évben az emberek millióit betegítik meg. Leggyakoribb virális okozóik: rhinovirusok (nátha), coronavírusok (nátha), respiratory syncytial vírus (bronchitis), influenzavírus (influenza), parainfluenzavírus (krupp), adenovírus (pharyngitis); leggyakoribb bakteriális okozóik: Staphylococcusok, Streptococcusok, Chlamydiák, Legionellák, Mycoplasmák (felső és alsó légúti megbetegedések, pneumonia). Nem rendelkezünk védőoltással sok szexuális úton terjedő fertőzéssel szemben sem, mint a *Neisseria gonorrhoeae* (gonorrhea), a *Treponema pallidum* (syphilis), a *Chlamydia trachomatis*-species tagjai (urethritis), a herpes simplex vírus 1-es és 2-es típusa (herpes labialis, herpes genitalis). Ugyancsak védtelenek vagyunk sok, az emésztőtraktust megbetegítő kórokozóval szemben, mint a hepatitis C-, D-, E-vírusok (hepatitis), rotavírusok (csecsemőkori hasmenés), Norwalk-vírus (gastroenteritis), adenovírusok (hasmenés). Nem tudunk védőoltással védekezni a congenitalis károsodást okozó cytomegalovirussal, a genitalis malignus daganatok etiológiai ágenseként számon tartott papillomavírusokkal, bizonyos lymphomák etiológiájában valószínűleg szerepet játszó Epstein–Barr-vírussal szemben. Nem lehet említés nélkül hagyni a humán immundeficiencia-vírus (HIV) elleni vakcina hiányát, amely kidolgozására pedig az elmúlt 15 évben világszerte igen nagy anyagi erőket mozgósítottak.

Úgy látszik, hogy vannak baktériumok és vírusok, amelyek ellen a konvencionális eljárásokkal (inaktiválás és attenuálás) nem lehet vakcinát kialakítani. Ennek oka ezen patogének tulajdonságaiban keresendő. Mint minden élőlénynek, így a patogén mikroorganizmusoknak is az elsődleges célja a túlélés.

Bizonyos vírusoknál és intracellulárisan szaporodó baktériumoknál olyan mechanizmusok alakultak ki, amelyek segítségével a fertőzés kiváltotta immunválasz elől el tudnak menekülni, azaz az immunválasz nem tudja őket elpusztítani.

Ilyen mechanizmusok például az intracelluláris perzisztencia/latencia, amelynek kapcsán a patogén genomja az akut fertőzés után hosszú ideig, esetleg az egész életén keresztül a gazdasejtben tartózkodik, de nem öli meg azt, hiszen ezzel önmagát is elpusztítaná. Nem világos, hogy ezen perzisztens fertőzések a gazdasejt milyen károsodását idézik elő, de krónikus idegrendszeri (Guillain–Barré-szindróma, sclerosis multiplex) és daganatos betegségek esetében (bizonyos lymphomák) perzisztens vírusfertőzések hatását feltételezik. A perzisztens fertőzések alatt az intracelluláris parazita genomja reaktiválódhat, és aktív produktív szaporodás indulhat el, amelynek kapcsán újabb sejtek fertőződhetnek, tehát a patogén túlélése biztosított. Az akut fertőzés után perzisztens/látens fertőzést alakítanak ki például az adeno-, a herpes- és a papillomavírusok *(7–9)*. Ezen patogének elleni immunvédelem kidolgozása azért nehéz, mert ezekben az esetekben nemcsak a patogén okozta megbetegedéstől kell megvédeni az egyént, hanem a fertőzéstől is, ami igen erős immunválasz esetén sem biztosított. Ilyen patogénekkel szemben az inaktivált vakcina nem nyújtana védelmet, élő attenuált vakcinával pedig a vakcinavírussal kialakítható perzisztens/látens fertőzés veszélye miatt nem tanácsos az immunizálás.

A patogének másik csoportjával történt fertőzés vagy vakcináció kivált ugyan protektív immunválaszt, de a kórokozó különböző törzsei közötti antigénkülönbségek (például a rhinovirusoknál) vagy a patogén felszíni antigénjeinek gyakori változása (például az influenzavírus és a HIV esetében) miatt az egyén védtelen az új antigénstruktúrával rendelkező patogénnel szemben, tehát újrafertőződik *(10–13)*.

Előfordul, hogy már az egyén szervezetén belüli szaporodás során megváltozik a patogén antigénstruktúrája (például HIV-fertőzésekben), így az egyén a reaktivációval szemben is védtelen.

A molekuláris biológia szemünk előtt lejátszódó hatalmas fejlődése új vakcinastratégiák kifejlődését tette lehetővé. Ezen stratégiák alapja a mikrobiális gének klónozása az expressziós vektorokba. Definíció szerint vektor az a mikroorganizmus vagy plazmid, amelynek genetikai állományába egy másik mikroorganizmus valamilyen génjét klónozták, amely gént a vektor sajátjaként fejez ki és termel. Vektorok lehetnek vírusok, baktériumok, gombák és bakteriális plazmidok. Ezen vektorokba klónozott gének produktumait vagy nagy mennyiségben termeltetik az expressziós vektorral, és tisztítás után használják immunizálásra, vagy magát az expressziós vektort inokulálják az immunizálandó egyénbe, és a beklónozott gén ott fejeződik ki, és váltja ki az immunválaszt. A kétféle felhasználás eredménye között lényeges különbség van: míg a tisztított produktum

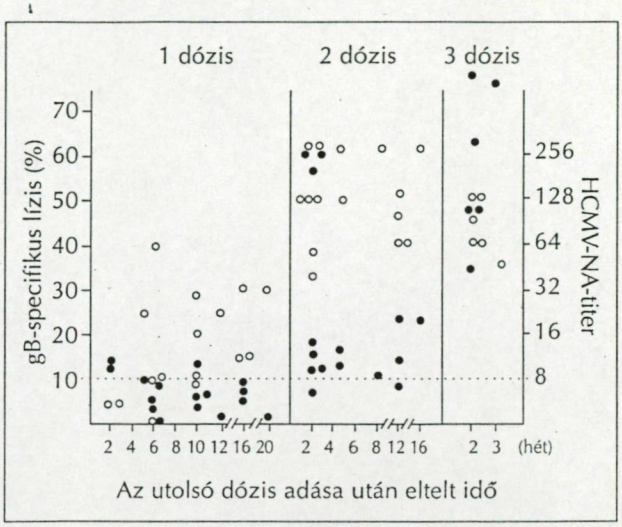
(fehérje) csak B-sejtes, azaz humorális immunválaszt tud kiváltani, addig a rekombináns vektor intracelluláris replikációja az immunizálandó organizmuson belül nemcsak humorális, hanem T-sejtes, azaz celluláris (például citotoxikus T-lymphocyta) immunválaszt is kivált. A humorális és a celluláris immunválasz együttes kialakulása és működése elengedhetetlen az intracellulárisan szaporodó organizmusok (vírusok, intracelluláris baktériumok) elleni védelemben.

Vírusvektorok

Érdekes módon nem sokkal azután, hogy a vacciniavírussal történő immunizálás eredményeként a WHO himlőmentesnek nyilvánította a Földet, vektorvírusnak a vacciniavírust javasolták *(14, 15)*. A vacciniavírus előnye, hogy egy nagyon sikeres vakcinációs programot hajtottak már végre vele, azaz a himlőmegbetegedést eradikálták, és hogy igen nagy méretű DNS-genommal rendelkezik, amelybe nagyméretű idegen DNS-szakaszokat, esetleg több protein kódolására alkalmas géneket tudnak beültetni anélkül, hogy a vírus struktúráját és funkcióját megzavarnák. Hátránya, hogy – bár nagyon ritkán, 100 000-ből egy esetben – komplikációkat, főleg encephalitist okoz az immunizált egyénnél. Továbbá, immunfunkciójukban károsodott egyének immunizálására sem javasolt az esetleg kialakuló generalizált vacciniamegbetegedés miatt.

Rekombináns konstrukciók inokulálásának feltétele természetesen a vektor ártalmatlan volta. A rekombináns vacciniavírusok potenciális káros hatásai miatt kidolgozott poxvírusvektor-alternatívákat egyrészt a további attenuálási folyamatokon átvitt vacciniavírus-törzsek, másrészt a madárpoxvírus-törzsek jelentették. A vacciniavírus további attenuálása itt azonban már nem empirikus volt, mint a korábbi vírusvakcinák attenuálására használt eljárások során, hanem a vacciniavírus-genom szerkezetének ismeretében a genomból egy 16 kilobázis hosszúságú génszakaszt eltávolítottak, amellyel a vírus replikációját gyengítették, de a korai promoterek alá beültetett idegen gének még kifejeződnek a vírussal fertőzött sejtekben. Ezen attenuált vacciniarekombinánsok állatkísérletekben a beültetett génproduktumra specifikus immunválaszt hoznak létre, de emberi immunizálásra jelen formájukban nem használják őket *(16)*.

Az emberi felhasználáshoz közelebb állnak a madárpoxvírus alapú rekombináns vírusok, ezek közül is a canaripox rekombináns vírusok. A kanárit megbetegítő poxvírus attenuált formáját használják vektornak, amely már a madarat sem betegíti meg, az emlőssejtekben pedig a teljes replikációra nem képes, de a korai génjeit kifejezi, tehát a korai promoterek alá ültetett idegen géneket is *(17–19)*.



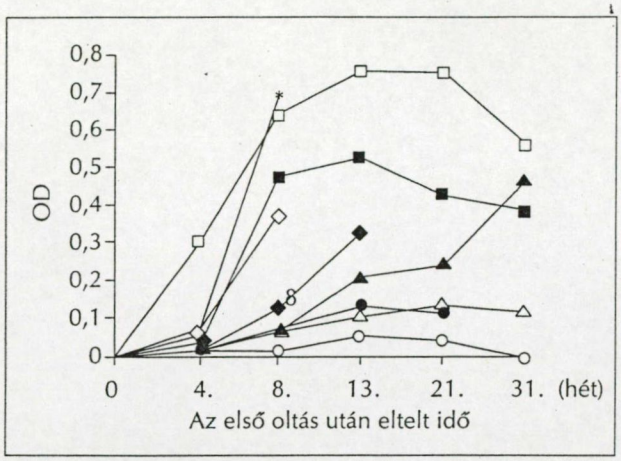
○ (HCMV-NA-titer): CMV-neutralizációs ellenanyagtiter (humorális immunválasz)

●: gB-specifikus lízis (citotoxikus T-sejt, azaz celluláris immunválasz)

p.f.u. (plaque forming unit): fertőző víruspartikula, amely szövetkultúra-sejtekben elszaporodva szemmel látható elváltozást, plakkot okoz

1. ábra. Immunizálás ALVAC-gB-vel CMV-specifikus neutralizáló ellenanyagot és citotoxikus T-sejt-választ indukál *(18). Három csoportba osztva összesen 58 egeret immunizáltak az ALVAC-gB rekombináns vírussal (sc. 1×10^6 p.f.u./dózis). Az első csoport egerei egy, a második csoport egerei kettő, a harmadik csoport egerei három oltásban részesültek. A CMV-gB-specifikus immunválaszt az ábrán jelölt időpontokban vizsgálták. Az adatok egyedi egerek immunválaszát tüntetik fel egy dózis alkalmazása után és két egér immunválaszának átlagát két vagy három dózis alkalmazása után.*

A kanáripox rekombináns vírusok protektív immunválaszt váltanak ki különböző emlősfajokban. Macskaleukémiavírus-fehérjét kifejező kanáripox-rekombinánssal immunizált macskák védettek voltak a macskaleukémiavírus-fertőzéssel szemben *(20)*, és kanyaróvírus-fehérjét kifejező kanáripox-rekombinánssal immunizált kutyák védettek voltak a kanyaróvírushoz hasonló antigénszerkezetű canine distemper vírus fertőzésével szemben. Ugyancsak a védelemhez elegendő szintű ellenanyagot váltott ki egy kanáripox-veszettség rekombináns vírus emberben *(20)*. Mint említettük, a beültetett idegen gén expressziója a vektorvírus DNS-szintézise előtt megtörténik, de a vektorvírus teljes replikációja gátolt a nem madáreredetű sejtekben, ami biztonságossá teszi az emlősszervezetek, így az ember immunizálását a kanáripox-rekombinánsokkal. Mivel a kanáripox-rekombinánsokkal inokulált egyének szervezetében a beültetett idegen gén kifejeződése intracellulárisan történik meg, a rekombinánsok mind humorális, mind celluláris immunválaszt kiváltanak. Ennek bizonyítása egy kanáripox-humán cytomegalovirus glikoprotein B (HCMV-gB)-rekombináns preklinikai kipróbálása során történt meg *(18)*. Az *1. ábra* mutatja, hogy egerek esetében – emberi inokulálásra alkalmas mennyi-



2. ábra. Immunizálás plazmid-gB-vel CMV-specifikus neutralizáló ellenanyagválaszt indukál *(24). Az egereket 50 μg plazmiddal immunizálták im. oltással, a negyedik és a nyolcadik héten újraoltásban részesültek. ELISA-val mérték a gB-specifikus ellenanyagokat tisztított gB-proteint használva antigénként. Az egyedi egerek savóinak OD-értékeit tüntetik fel a görbék, a savók 1:40 hígításúak.*

ségben ($1{,}5\times10^6$ p.f.u.) adva – egy dózis után alacsony ellenanyagszint és a kimutathatóság küszöbértéke alatti citotoxikus T-lymphocyta (CTL)-válasz, két dózis után magasabb szintű ellenanyag és kimutatható szintű CTL-válasz, a harmadik oltás után az előzőhöz hasonló ellenanyagszint és magas szintű CTL-válasz mérhető, ami a rekombináns vírus jó hatásfokú insert-specifikus immunogenitását mutatja, és azt a feltételezést bizonyítja, hogy kanáripox-rekombinánssal történő immunizáláskor mind humorális, mind CTL-válasz kialakul. Mivel az insertspecifikus ellenanyagválasz nem volt magas a második dózis inokulálása után, és nem fokozódott a harmadik inokulálást követően *(1. ábra)*, felmerült, hogy specifikus fehérjeantigénnel végzett ismételt oltással esetleg nagyobb ellenanyagválaszt lehet kiváltani, azaz a kanáripox-rekombináns jó „priming" hatású (az elsődleges immunválasz lassabban fejlődik ki és kisebb, mint az azt követő, miközben memóriasejtek alakulnak ki), de kevésbé hatékony „booster" hatású (az ismételt antigén-találkozáskor gyorsabb és intenzívebb választ kiváltó) vektorkonstrukció. Egérimmunizálási kísérletek igazolták, hogy a kanáripox-HCMV-gB rekombináns vírussal végzett első oltás után a második oltásként alkalmazott tisztított fehérje igen magas ellenanyagszintet váltott ki az immunizált állat szervezetében. A kanáripox-rekombináns „priming" hatását az egérlymphocyták specifikus stimuláció utáni interferon-γ-termelése is jelezte, amely a celluláris immunválasz irányában elkötelezett T-sejtek jelenlétét igazolta. Az interferon-γ antivirális hatással is rendelkezik, így a védőhatás egyik komponense is lehet.

Mivel a felnőtt populáció (az 1980 előtt született egyének) a himlőmegbetegedéssel szembeni védelem céljából immunizálva lett vacciniavírussal, kérdés volt, vajon a vacciniaimmunitás nem fogja-e gátolni a vacciniával rokon kanáripoxvírussal való immunizálás hatásfokát. Ezért egereket immunizáltak vacciniavírussal, és a vacciniaimmunizált egereket később immunizálták a kanáripox-HCMV-gB rekombináns vírussal. Az eredmények azt igazolták, hogy a vacciniaimmunitás nem gátolja a kanáripox-rekombinánssal kiváltható insert-specifikus immunválaszt, tehát vaccinia-védőoltásban részesült emberek is immunizálhatók kanáripox-rekombinánsokkal. A kanáripox-HCMV rekombináns vírusok humán kipróbálás alatt vannak.

Vektorvírusnak nemcsak poxvírusokat, hanem genetikailag modifikált adenovírusokat, herpes simplex vírust, retrovírusokat is javasolnak. Különösen a modifikált adeno- és retrovírusvektorok alkalmazhatók potenciálisan bizonyos genetikai betegségek (például cysticus fibrosis) terápiájára is, azaz a hiányzó vagy hibás sejti gének funkcionálisan ép génekkel való pótlására, ezáltal a génhiányos állapot tüneteinek megszüntetésére. Ezen hibás sejti géneket pótló rekombináns vakcinák a rekombinánsok felhasználásának célját is kiszélesítették, azaz nemcsak fertőző betegségek megelőzésére, hanem genetikai betegségek gyógykezelésére is alkalmazhatók *(21)*.

Baktériumvektorok

Baktériumvektorként elsősorban attenuált Salmonella-törzsek és BCG jönnek szóba. A Salmonella-vektor előnye, hogy orálisan alkalmazva a gastrointestinalis rendszerben szaporodik, és mucosalis immunitást vált ki a bélrendszerben, így az enteralis betegségek kórokozóinak megfelelő génjeit beültetve ezen enteralis betegségek megelőzésére lenne alkalmas. A BCG-vektor előnye, hogy a születés után nem sokkal adva vakcinaként kiterjedten alkalmazzák, valamint celluláris immunválaszt vált ki, így intracellulárisan szaporodó mikroorganizmusok (vírusok és intracellulárisan szaporodó baktériumok, paraziták) elleni védelemben hasznos lehet.

Genetikai vagy DNS-immunizálás, azaz expressziós plazmidvektorok

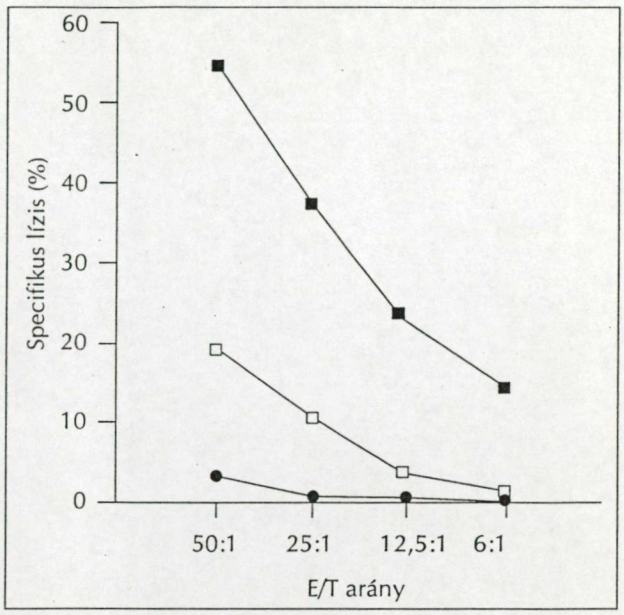
Az alap- és alkalmazott immunológiai tudományok nagy meglepetése volt az 1990-es években annak felismerése, hogy az izomba vagy bőrbe inokulált DNS az általa kódolt fehérjét kifejezve immunogén, és immunválaszt vált ki. A technológia lényege az, hogy az immunogén fehérje génjét bakteriális plazmidba kódolják az eukaryotában funkcionáló promoter és enhancer szekvenciák alá. Az ilyen módon elkészített plazmid-DNS-t izomba vagy bőrbe adva a génpro-

duktum (fehérje) hosszú ideig tartó helyi kifejeződése jön létre, amely hosszú ideig tartó immunválaszt eredményez. Az immunválasz mind B-, mind T-sejtes, azaz ellenanyagok és celluláris immunválasz jön létre. A celluláris immunválaszt a citotoxikus T-sejtek indukciója is jellemzi, tehát a fehérje intracellulárisan fejeződik ki, és az MHC I. osztály molekulái mutatják be az antigén-prezentáló sejtek felszínén a T-sejtek részére. A plazmidvektorok előnye, hogy hosszú ideig tartó immunválaszt eredményeznek, az immunválasz mind B-, mind T-sejtes, és mivel fertőző ágens bevitele nem történik az immunizáció kapcsán, ismételt dózis immunogenitását nem akadályozza az előző dózisra kiváltott vektorspecifikus immunválasz. További előnyük, hogy előállításuk egyszerű és olcsó *(22–24)*.

A *2. ábra* szemlélteti, hogy egy expressziós eukaryotaplazmid, amelybe a HCMV-gB gént klónozták, egerek esetében gB-specifikus ellenanyagválaszt vált ki; a plazmid kétszeri vagy háromszori im. oltása után az egerek többségénél tartósan (a vizsgálat közel nyolc hónapja alatt) tapasztalható magas szintű ellenanyagválasz. Az egerek vérsavójában HCMV neutralizáló ellenanyagokat is kimutattak.

A *3. ábra* azt mutatja, hogy egy expressziós eukaryotaplazmid, amelybe a HCMV-65 kD foszfoprotein gént (pp65) klónozták, pp65-specifikus ellenanyagválaszt indukált egerek esetében kétszeri im. oltás után. Hasonlóan a humorális immunválaszhoz a plazmid pp65-specifikus CTL-választ is kiváltott az egereknél. A két plazmid kevert inokulálásakor mind gB-, mind pp65-specifikus ellenanyagok és CMV-neutralizáló aktivitást mértek a savókban. Ugyanezen egerek T-lymphocytái pp65-specifikus CTL-aktivitással rendelkeztek. Ezek az eredmények alátámasztják a DNS-immunizálás lehetőségét CMV okozta megbetegedések kivédésére is *(24)*.

Potenciális hátrányuk például a gazdasejt genomjába való integrálódás, amely a gazdasejt genetikai változását idézheti elő. A plazmidvektorok tanulmányozása világszerte intenzíven folyik, azonban integrálódást a gazdasejtbe vagy a DNS-immunizálás egyéb esetleges komplikációját eddig nem tapasztalták. A legtöbb vírus protektív immunválaszban részt vevő génjét plazmidvektorba már beklónozták, és kísérleti állatok esetében bizonyították a hatásosságát. Expressziós eukaryotaplazmidokba klónozott HIV-génekkel történő emberi immunizálási próbálkozások folyamatban vannak.



■ = *Vaccinia-pp65 rekombinánssal fertőzött MCH-I. osztály szerint megegyező sejtek*
□ = *Szülő (nem rekombináns) vacciniavírussal fertőzött MHC-I. osztály szerint megegyező sejtek*
● = *Vacciniavírus-pp65 rekombinánssal fertőzött, MHC-I. osztály szerint különböző sejtek*

3. ábra. Immunizálás plazmid-pp65-tel CMV-specifikus citotoxikus T-lymphocyta-választ indukál *(24). Az egereket a 2. ábránál leírt módon immunizálták, és az utolsó oltás után egy hónappal ölték le. A lépsejteket pp65-specifikusan stimulálták in vitro, és azonos, valamint különböző MHC-I. osztályba tartozó sejtekben mérték a citotoxicitást. Az ábra egy reprezentatív egér esetében kapott eredményeket mutatja. A szülő-vacciniavírussal fertőzött MHC-I. szerint megegyező sejtek és a vaccinia-pp65 rekombináns vírussal fertőzött MHC-I. megegyező sejtek lízise közötti különbség mutatja a pp65-specifikus lízist.*

Szubunit vakcinák

Ha ismert, hogy a vírus vagy baktérium melyik alkotóeleme váltja ki a protektív immunválaszt, ezen alkotórészt vakcinaként lehet használni. Erre példa a hepatitis B-vírus felszíni antigénje (HBsAg), amely nagy mennyiségben termelődik a vírussal fertőzött sejtekben, és tisztított állapotban előállítható a vírushordozó emberek plazmájából, vagy mostanában rekombináns DNS-technológia segítségével. Az utóbbi eljárással előállított HBsAg-vakcinát használják jelenleg immunizálására. Több más vírus protektív hatásáért felelős fehérjéjét is előállították már a rekombináns DNS-technológia módszerével (például herpes simplex vírus 2-es típusa, cytomegalovirus), azonban ezek immunogenitása gyengének bizonyult. A szubunit vakcinák immunogenitásának növelése új adjuvánsok és az immunogenitást fokozó más eljárások kidolgozásának segítségével világszerte folyik.

A szubunit vírusvakcinák közé sorolhatók a szintetikus úton előállított peptidek, amelyek B- vagy T-sejt-epitopként viselkednek természetes környezetükben, a vírusproteinben. Előnyük, hogy a protein nemkívánatos részeit nem tartalmazzák. A szintetikus peptidek azonban általában gyengén immunogének. Immunizálásra használhatók, ha a peptideket hordozó molekulákhoz kötik, és adjuvánssal együtt adják az

1. táblázat. 2025-ig valószínűleg bevezetésre kerülő új vagy a jelenleginél fejlettebb vakcinák

Vírusvakcinák
Influenzavírus*
Hepatitis A vírus*
Parainfluenzavírus
Respiratory syncytial vírus
Adenovírus 1, 2, 5–7 típusok
Rotavírus
Hepatitis C-, E-vírus
Humán immundeficienciavírus
Papillomavírus
Herpes simplex vírus 2. típus
Cytomegalovirus
Epstein–Barr-vírus
Baktérium- és parazitavakcinák
Pneumococcus*
Meningococcus*
*Mycobacterium tuberculosis**
*Vibrio cholerae**
Acelluláris pertussis
E. coli (orális enterotoxinogén)
Helicobacter pylori (ulcus duodeni, ventriculi)
Borrelia burgdorferi (Lyme-kór)
Malária

** Jelenleg is meglévő, fejlesztésre kerülő vakcinák*

immunizálandó egyénnek. Több B- és T-sejt-epitop összekapcsolásával hatásosabb vakcina állítható elő. Több T-sejt-epitop összekapcsolása esetén is előfordulhat, hogy az egyén HLA 1-es és 2-es osztályba tartozó molekulái nem hordoznak olyan allélokat, amelyek a szintetikus T-sejt-epitopokat tálalni tudják a T-sejtek felé, így az ilyen egyén esetében immunválasz nem jön létre. A szintetikus peptidvakcinák alkalmasak lehetnek vakcinációra, ha a leírt problémák megoldásra kerülnek. Vírusbetegségek profilaxisára még nem használják őket.

Néhány további fontos és nem kellően ismert aspektusa a vakcináció tudományának:

1. Újszülöttek immunizálásának problémái, amelyet az újszülött immunrendszerének működésére vonatkozó igen hiányos ismereteink hátráltatnak.

2. A mucosalis immunitást kiváltó eljárások kezdetlegesek, amelyek pedig igen fontosak lennének, hiszen sok patogén (HIV-, influenza-, herpesvírus) behatolási helye elsősorban a mucosalis hártya, így a szisztémás immunitás mellett a lokális, mucosalis immunitás is szükséges lenne.

3. Egyetlen dózisban adott, több immunogént tartalmazó vakcina lehetősége, amely nemcsak azon múlik, hogy például melyik vektorba lehet többféle fehérjét kódoló gént beültetni, hanem ezen immunogének esetleges immunológiai kompetíciójának ismeretére is szükség lenne.

4. Bizonyos patogének (főleg paraziták) patológiájáért felelős immunológiai reakciók megértésének hiányosságai.

A vakcinológia mostanihoz hasonló nagy lendületű fejlődése azonban további izgalmas és hasznos eredményeket fog produkálni a fent vázolt problémakörökben is.

A világszerte folyó kutatómunkák alapján a következő 25-30 évben az *1. táblázatban* felsorolt új vagy a jelenleginél fejlettebb vakcinák használatba lépése jósolható meg *(20)*.

Meghaladná a dolgozat kereteit annak a megbeszélése, hogy a felsorolt vakcinációs próbálkozások közül az előre jelzett vakcinák mindegyikére vonatkozóan melyik bizonyul optimálisnak, mivel ez a patogén antigénjeitől és a betegség patomechanizmusától is függ.

A vakcinológia a dolgozatban érintett mikrobiológiai, immunológiai, molekuláris biológiai kérdéscsoportokat öleli fel, és napjainkban már önálló tudományágként működik. Új kórokozók felbukkanása (HIV), régi kórokozókkal kiváltott betegségek újra megjelenése (tbc), vagy annak a felismerése, hogy klasszikus belgyógyászati betegségeket fertőző ágensek okozhatnak (*Helicobacter pylori*-baktérium az ulcus duodeni és ventriculi eseteinek nagy részét), a vakcinológia tudományát fontossá és aktuálissá teszik.

A közlemény az MKM 528, az AMFK 474 és az OTKA T 19 256 számú pályázatának támogatásával készült.

IRODALOM

1. *Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID.* Smallpox and its eradication. *Geneva, Switzerland: World Health Organization, 1988.*
2. *Johan Béla Országos Epidemiológiai Központ.* Oltóanyagok: 1997-ben törzskönyvezett oltóanyagokkal kapcsolatos részletesebb információk. I. Hiberix (*Haemophilus influenzae* B konjugált vakcina). *Epinfo 1998;5:1–8.*
3. *Johan Béla Országos Epidemiológiai Központ.* Oltóanyagok. II. 1997-ben törzskönyvezett oltóanyagokkal kapcsolatos részletesebb információk. Hepatitis A elleni vakcinák. *Epinfo 1998;5:13–24.*
4. *Johan Béla Országos Epidemiológiai Központ.* AIDS elleni oltóanyag kérdésköre. *Epinfo 1998;5:430–431.*
5. *Johan Béla Országos Epidemiológiai Központ.* Módszertani levél az 1999. évi védőoltásokról. Életkorhoz kötötten kötelező védőoltások. *Epinfo 1999;1:10–20.*
6. *Burnette WN, Samal B, Browbe JK, Fenton D, Bitter GA.* Production of hepatitis B recombinant vaccines. *Cold Spring Harbor, NY: Cold Pring Harboe Laboratory 1984:245–250.*
7. *Horwitz MS.* Adenoviruses. In: *Fields.* Virology. 3rd ed. *Lippincott–Raven Publ, 1996:2149–2171.*
8. *Banks TA, Rouse BT.* Herpesviruses-immune escape artists? *Clin Infect Dis 1992;14:933–941.*
9. *Shah KV, Howley PM.* Papillomaviruses. In: *Fields.* Virology. 3rd ed. *Lippincott–Raven Publ, 1996:2077–2109.*

10. *Couch RB.* Rhinoviruses. In: Fields. Virology. 3rd ed. *Lippincott–Raven Publ, 1996:713–734.*
11. *Murohy BR, Webster RG.* Orthomyxoviruses. In: Fields. Virology. 3rd ed. *Lippincott–Raven Publ, 1996:1397–1445.*
12. *Hirsch MS, Curran JW.* Human immunodeficiency viruses. In: *Fields.* Virology. 3rd ed. *Lippincott–Raven Publ, 1996:1953–1975.*
13. *Norley S, Kurth R.* Vaccination against HIV. *Immunobiology 1992;184:193–207.*
14. *Mackett M, Smith GL, Moss B.* Vaccinia virus: a selectable eukaryotic cloning and expression vector. *PNAS USA 1982;79:7415–7419.*
15. *Panicali D, Paoletti E.* Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. *PNAS USA 1982;79:4927–4931.*
16. *Moss B.* Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *PNAS USA 1996;93: 11341–11348.*
17. *Taylor J, Weinberg R, Tartaglia J, et al.* Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoprotein. *Virology 1992;187:321–328.*
18. *Gönczöl E, Berencsi K, Pincus S, Endresz V, Méric C, Paolettie E, et al.* Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate. *Vaccine 1995;13:1080–1085.*
19. *Gönczöl E, Berencsi K, Kaufíman E, Endresz V, Pincus S, Cox W, et al.* Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate; immune response elicited in a prime/boost protocol with the glycoprotein B subunit. *Scand J Infect Dis Suppl 1995;99:110–112.*
20. *Plotkin SA.* Vaccination in the 21st century. *Infectious diseases 1993;168:29–37.*
21. *Rosenfeld MA, Yoshimura K, Trapnell BC, et al.* In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell 1992;68:143–155.*
22. *McCarthy M.* DNA vaccination: a direct line to the immune system. *The Lancet 1996;348:1232.*
23. *Robinson HL.* Nucleic acid vaccines: an overview. *Vaccine 1997;15:785–787.*
24. *Endresz V, Kari L, Berencsi K, Kari Cs, Gyulai Zs, Jeney Cs, et al.* 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;17:50–58.*

VII.

LEGE ARTIS MEDICINÆ

ÚJ MAGYAR ORVOSI HÍRMONDÓ

ALAPÍTVA 1990-BEN A MAGYAR ORVOSLÁS TUDOMÁNYOS ÉS MŰVÉSZI SZÍNVONALÁNAK EMELÉSÉRE, A NEMZET EGÉSZSÉGI ÁLLAPOTÁNAK JOBBÍTÁSÁRA.

KÜLÖNLENYOMAT

Szisztémás retinoidok

A 9-cis-retinoidsav a betegek 37%-ánál klinikai választ váltott ki, míg 34%-uk stabil maradt *(36)*. *Saiag* mucocutan Kaposi-sarcomában all-trans-retinoidsavval 40%-os klinikai választ ért el, emellett a betegek 40%-a nem progrediált a vizsgálati időszakban *(37)*.

Humán choriogonadotropin

Harris valamennyi betegénél tumorregressziót ért el 150 000 egység HCG emelkedő dózisú alkalmazásával, *Tavio* ugyanakkor nyolc betege közül csak egynél észlelt részleges javulást intramuscularisan adott 100 000–300 000 egységek alkalmazásával *(38)*.

IRODALOM

1. *Chang Y, Cesarman E, et al.* Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science 1994;266:1865-9.*
2. *Ambroziak JA, et al.* Technical comments: Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. *Science 1995;268:582-3.*
3. *Moore PS, Chang Y.* Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma patients with and without HIV-infection. *N Engl J Med 1995;332:1181-5.*
4. *Moore PS, et al.* Molecular mimicry of human cytokine and cytokine response pathway genes by HHV-8. *Science 1996;274:1739-44.*
5. *Renne R, et al.* Lytic growth of Kaposi' sarcoma-associated herpesvirus (human herpesvirus-8) in culture. *Nature 1996;2:342-6.*
6. *Foreman KE, et al.* Propagation of a human herpesvirus from AIDS-associated Kaposi's sarcoma. *New Engl J Med 1997;336(3):163-71.*
7. *Russo JJ, et al.* Nucleotide sequence of Kaposi's sarcoma-associated hepesvirus (HHV-8). *Proc Nat Acad Sc 1996;93:14862-7.*
8. *Cesarman E, Chang Y, et al.* Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N Eng J Med 1995;332:1186-91.*
9. *Schulz TF.* Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8). *J Gen Virol 1998;79:1573-91.*
10. *Kemény L, Gyulai R, et al.* Kaposi-sarcomához asszociált herpesvirus: human herpesvirus 8. *Orvosi Hetilap 1997;138(16):979-83.*
11. *Gyulai R, Kemény L, et al.* Herpesvirus-like DNA sequence in angiosarcoma in patient without HIV infection. *New Eng J of Med 1996;334(8):540-1.*
12. *Poole LJ, Zong J, et al.* Comparison of genetic variability at multiple loci across the genomes of the major subtypes of Kaposi's sarcoma-associated herpesvirus reveals evidence for recombination and for two distinct types of open reading frame K15 alleles at the right-hand end. *J Virol 1999;738:6646-60.*
13. *Lennette ET, et al.* Antibodies to human herpesvirus type 8 in the general population and in Kaposi's sarcoma patients. *Lancet 1996;348(9031):858-61.*
14. *Juhász A, Remenyik É, et al.* Humán herpesvírus-8-specifikus antitestek előfordulása magyar véradókban és Kaposi-sarcomás betegekben. *Orv Hetilap 1998;139(50):3001-4.*
15. *Nagy K, Kemény B, et al.* Kaposi-sarcomához társult humán herpesvírus-ellenanyagok HIV-fertőzött és egészséges magyar populációban. *Magyar Venerol Arch 1998;II(1):17-20.*
16. *Mendez JC, Paya CV.* Kaposi's sarcoma and transplantation. *Herpes the J IHMF 2000;1(7):18-23.*
17. *David S, MacDougall.* The international symposium on HIV, leukemia and opportunistic cancers, Marrakech, Morocco. *Journal of IAPAC 1999;5(9):48-55.*
18. *Gao, et al.* Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus related latent nuclear antigens before the development of Kaposi sarcoma. *New Engl J Med 1996;335:233-41.*
19. *Martin JN, et al.* Sexual transmission and the natural history of human herpesvirus 8 infection. *New Engl J Med 1998;338(14):948-54.*
20. *Levy JA.* Three new human herpesviruses (HHV6, 7, and 8). *Lancet 1997;349:558-62.*
21. *Whitby D, Howard MR, et al.* Detection of Kaposi's sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. *Lancet 1995;346:799-802.*
22. *Lock MJ, Griffiths PD, Emery VC.* Development of a quantitative competitive polymerase chain reaction for human herpesvirus 8. *J Virol Methods 1997; 64:19-26.*
23. *Shepherd FA, Maher E, Cardella C.* Treatment of Kaposi sarcoma after solid organ transplantation. *J Clin Oncol 1997;15:2371-7.*
24. *Medve, et al.* Long term experience with liposomal doxorubicin as treatment for AIDS-related Kaposi sarcoma. *4th Conf Retro and Opportun Infect, 1997.*
25. *Northfelt DW, et al.* Pegylated doxorubicin versus doxorubicin, bleomycin, and vincristine in the treatment of AIDS-related Kaposi sarcoma: results of a randomized phase III clinical trial. *Journal Clin Oncol 1998;244:2451.*
26. *Nasti G, et al.* Evidence of activity of vinorelbine in patients with previously treated AIDS associated Kaposi sarcoma. *The Third International Congress on Drug Therapy in HIV-infection. 1996. Abstract OP5.*
27. *Dezube BJ, et al.* Fumagillin analogue (TNP-470) in the treatment of Kaposi sarcoma: a phase I AIDS Clinical Trials Group Study *National AIDS Malignancy Conference 1997. Abstract 78.*
28. *Podzamczer D, et al.* Alpha-interferon (IFN) plus zidovudine in Kaposi sarcoma: a randomized trial comparing IFN 3 MU vs. 10 MU daily Int Conf AIDS 1996;11:303.
29. *Kedes DH, Ganem D.* Sensitivity of Kaposi sarcoma-associated herpesvirus replication to antiviral drugs. *J Clin Invest 1997;99(9):2082-6.*
30. *Looney DJ, et al.* Utility os HHV-8 DNA QC PCR: relationship of PBMC burden to stage of Kaposi sarcoma and response to therapy. *5th Conf Retrovir Opport Infect 1998;17. Abstract 525.*
31. *Low P, et al.* Suppression of HHV-8 viremia by foscarnet in an HIV-infected patient with Kaposi sarcoma and HHV-8 associated hemophagocytic syndrome. *Eur J Med Res 1998;10:461-4.*
32. *Robles R, et al.* Effect of antiviral drugs used to treat cytomegalovirus and-organ disease on subsequent course of previously diagnosed Kaposi sarcoma in patients with AIDS. *J Acquir Immune Defic Syndr Hum Retrovirol 1999;20(1):34-8.*
33. *Mastroianni CM, et al.* Regression of AIDS-related Kaposi sarcoma lesions following treatment with cidofovir. *Int Conf AIDS 1998;12:1059.*
34. *Simonart T, et al.* Treatment of classical Kaposi sarcoma with intralesional injection of cidofovir report a case. *J Med Virol 1998;3:215-8.*
35. *Nunez, et al.* Presence of HHV-8 viremia in HIV-infected patients with Kaposi sarcoma treated with liposomal doxorubicin. *ICAAC 1999;9:113.*
36. *Miles S.* Antitumor activity of oral 9-cis-retinoic acid in AIDS related Kaposi's sarcoma. *12th World AIDS Conf, Geneva, 1998. Abstract 22276.*
37. *Saiag P, et al.* Treatment of AIDS-related Kaposi sarcoma with all trans-retinoic acid. *Eleventh Conf AIDS 1996;2220.*
38. *Tavio M, et al.* Human chorionic gonadotropin in the treatment of HIV-related Kaposi sarcoma. *Eur J Cancer 1998;10:1634-7.*

A herpesvírusok elleni vakcináció lehetőségei

Gyulai Zsófia

POSSIBILITIES OF VACCINATION AGAINST HERPESVIRUS DISEASES

Currently available vaccines provide protection against various infectious diseases, but there is an even longer list of infections that can not yet be prevented by vaccination, including the majority of diseases caused by herpesviruses (with the exception of varicella). Herpesviruses are widespread in the population, and although most infections are asymptomatic or manifest in clinically mild symptoms, there are severe, often life threatening diseases seen in the immunocompromized patients and in neonates. The complex interplay between host and virus makes it difficult to mount useful vaccine strategies. Herpesviruses have developed a number of successful mechanisms to evade immunosurveillance. Following acute infection they establish latency, during which they express few, if any protein antigens. In addition, herpesviruses interfere with cytotoxic T-cell function and antigen recognition by different means. An ideal herpesvirus vaccine would not only prevent disease development, but also the infection and the establishment of latency, which is not easy to achieve, since even natural immunity does not provide complete protection. Conventional vaccines (containing attenuated live or inactivated viruses) do not afford protection against these viruses, nor their use would meet the necessary safety requirements. The use of gene technology has opened up an exciting new era in vaccinology, providing alternative possibilities for the development of effective and safe new vaccines. Examples of these alternative possibilities include recombinant vaccines and DNA-immunization.

herpes simplex viruses, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, recombinant vaccines, DNA-immunization

A jelenleg forgalomban lévő vakcinák védelmet biztosítanak számos fertőző betegséggel szemben, azonban rendkívül sok fertőzés kialakulását még ma sem tudjuk védőoltással megakadályozni. A bárányhimlőt kivéve ide sorolhatók a herpesvírusok által okozott megbetegedések is. A herpesvírusok igen elterjedtek a populációban, és noha klinikailag a fertőzés általában tünetmentes vagy enyhe lefolyású, csökkent immunfunkciójú egyéneknél és újszülötteknél súlyos, gyakran életveszélyes megbetegedések fordulnak elő. A megfelelő vakcinációs stratégia kialakítását megnehezíti a gazdaszervezet és a vírus közötti összetett kölcsönhatás. Különböző mechanizmusok segítségével a herpesvírusok rejtve maradhatnak a fertőzés kiváltotta immunválasz elől. A herpesvírusok az akut fertőzést követően latenciát alakítanak ki, amelynek kapcsán a patogén genomja hosszú ideig a gazdasejtben tartózkodik, és a latens fertőzés alatt csak néhány gén transzkripciója, esetleg transzlációja megy végbe. A herpesvírusok többféle módon gátolják az adaptív immunválaszban szerepet játszó citotoxikus T-lymphocyták antigénfelismerő és a fertőzött sejtet pusztító hatását is. A hatékony immunvédelem kialakítása azért is nehéz, mert nemcsak a herpesvírus okozta megbetegedéstől kellene megvédeni a szervezetet, hanem a fertőzéstől, így a latencia kialakulásától is; erre azonban a természetes fertőzés által kialakított immunválasz sem képes. Ezen megbetegedésekkel szemben a konvencionális úton előállított vakcinák (élő, attenuált, illetve inaktivált vírusok) nem biztosítanak kellő védelmet, vagy alkalmazásuk nem biztonságos. A géntechnológia alkalmazása új, izgalmas korszakot nyitott a vakcinagyártás területén, lehetővé téve hatékony és biztonságos oltóanyagok kifejlesztését. Ezen új, alternatív megoldások közé tartoznak a rekombináns vakcinák és a DNS-immunizálás.

herpes simplex vírusok, varicella-zoster vírus, cytomegalovirus, Epstein–Barr-vírus, rekombináns vakcinák, DNS-immunizálás

DR. GYULAI ZSÓFIA (levelező szerző/correspondent): Szegedi Tudományegyetem, Általános Orvostudományi Kar, Orvosi Mikrobiológiai Intézet/University of Szeged, Faculty of Medicine, Department of Medical Microbiology; H-6720 Szeged, Dóm tér 10.

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A vakcináció az orvostudomány legnagyobb felfedezései közé tartozik. *Jenner* korszakalkotó megfigyelése után, amely a himlő elleni sikeres vakcinációhoz és az 1970-es évek végére a Föld himlővírus-mentesítéséhez vezetett, 80 év telt el, míg *Pasteur* létrehozta a veszettség megelőzésére az első élő, attenuált kórokozót tartalmazó vakcinát. Nem sokkal ezután *Salmon* és *Smith* elölt Salmonella baktériumokkal tudott immunizálni. Így már több mint száz éve ismert az immunizálás két fő alapmódszere, az élő, attenuált mikroorganizmusokkal és az inaktivált, teljes kórokozókkal végzett vakcináció. A tisztított, egyedi mikrobiális antigénkomponensek immunogenitásának felismerése megteremtette az alegységvakcinák bevezetésének elméleti alapját. Az oltóanyagok ezen típusába sorolhatók a tokos baktériumok tisztított poliszacharid-antigénjeit tartalmazó készítmények is. A molekuláris biológiai módszerek fejlődésével lehetővé vált a mikrobiális gének klónozása különböző vektorokba (vírusok, baktériumok, gombák, növények), és az ezek által termeltetett baktérium- vagy vírusfehérjéket használják fel immunizálás céljára. A tisztított fehérjék általában humorális immunválaszt hoznak létre; a rekombináns expressziós vektorok (baktériumok, vírusok és plazmidok) felhasználásával végzett vakcinálás azonban úgy celluláris, mint humorális immunválaszt eredményez.

A herpesvírusok családjába tartozó több mint 100 különböző vírus közül jelenlegi ismereteink szerint nyolcat sorolunk az embert megbetegítő kórokozók közé. Ezen nagyméretű, borítékos, kettős szálú, lineáris DNS-genommal rendelkező vírusok által okozott megbetegedések széles spektrumot alkotnak; jellemző tulajdonságuk, hogy a primer fertőzést követően a gazdaszervezetben latenciát alakítanak ki, amelyet a vírus periodikus reaktivációi követnek. A herpesvírusok igen elterjedtek, a populáció nagy része fertőződik velük és immunitással rendelkezik. Klinikailag a fertőzés általában enyhe vagy tünetmentes formában zajlik, de csökkent immunfunkciójú egyéneknél súlyos, gyakran életveszélyes megbetegedések fordulnak elő.

A herpesvírusokat a szaporodási ciklus hossza, a sejtkárosító hatás és a latens fertőzés jellemzői alapján három (α-, β- és γ-herpesvírus) alcsaládba sorolják. Az α-herpesvírusok közé tartozik a herpes simplex vírus 1-es és 2-es típusa (HSV-1, HSV-2) és a varicella-zoster vírus (VZV), amelyek érző ganglionokban alakítanak ki latens fertőzést. A cytomegalovirus (CMV) β-herpesvírus; latens módon valószínűleg a monocyta eredetű sejtek és a nyálmirigy, valamint a vesetubulusok epithelialis sejtjei hordozzák. A lymphotrop humán herpesvírus-6-ot (HHV-6) és humán herpesvírus-7-et (HHV-7) szintén a β-herpesvírusok közé sorolják. A γ-herpesvírusok közé tartozó Epstein–Barr-vírus (EBV) az oropharynx és a parotis epithelialis sejtjeit, valamint a B-lymphocytákat fertőzi meg, és a B-lymphocytákban alakít ki latenciát. Szintén γ-herpesvírus a humán herpesvírus-8 (HHV-8). Az utóbbi két vírusnak kóroki szerepet tulajdonítanak bizonyos humán malignus daganatok kialakulásában. A humán patogén herpesvírusok által okozott megbetegedéseket és a latencia jellegzetes anatómiai lokalizációit az *1. táblázatban* foglaltuk össze.

Az élethosszig tartó fertőzöttség, valamint az immunhiányos állapotokban fellépő betegség és az újszülötteknél észlelhető súlyos kórlefolyás miatt szükséges a hatékony vakcina mielőbbi kifejlesztése. Az EBV- és a HHV-8-fertőzések megelőzése vakcinációval elősegíthetné az általuk okozott rosszindulatú daganatos megbetegedések előfordulásának csökkenését is.

A gazdaszervezet és a vírus közötti komplex kölcsönhatás nagymértékben lassítja a megfelelő vakcinációs stratégia kialakítását. Mindegyik herpesvírus latens fertőzést hoz létre, és ezen idő alatt csak néhány gén transzkripciója, esetleg transzlációja megy végbe; ezenkívül többféle mechanizmus révén gátolják az adaptív immunválaszban szerepet játszó citotoxikus T-lymphocyták (CTL) antigénfelismerő és sejtpusztító hatását, így blokkolják a vírusantigének prezentációját az MHC I hisztokompatibilitási antigénekkel *(1)*. Mindezen hatásoknak köszönhetően a vírus rejtve maradhat az immunrendszer számára. Ismeretes továbbá, hogy a természetes fertőzés által kialakított immunvédelem csak részben védi meg a szervezetet az újrafertőződéstől, és az immunrendszer reaktivációk alkalmával létrejövő restimulációja nem jelent tökéletes védelmet az újbóli reaktivációkkal szemben. Ezen adatok birtokában tehát nehéz meghatározni a hatékony herpesvírus-vakcinával szembeni elvárásokat. Olyan törekvés látszik reálisnak, amely csökkentené a fertőzés helyén a replikálódó vírusok mennyiségét, így enyhítené az akut fertőzés tüneteit, csökkentené a latensen fertőzött sejtek számát, és így a reaktivációt is. A különböző herpesvírusokkal kapcsolatosan részletesebben tanulmányozott vakcinákat a *2. táblázatban* foglaltuk össze.

Herpes simplex vírusok

A többi herpesvírushoz hasonlóan a herpes simplex vírusok is igen elterjedtek a populációban. Széles gazdaspektrummal rendelkeznek, gyorsan szaporodnak és erősen citolitikus hatásúak. A két herpes

nák felhasználásánál számos előny adódhat. A herpes simplex vírusok által okozott fertőzések megelőzésében hatékonysága az élő, attenuált vakcináéhoz hasonlítható. Ezen oltóanyagok alkalmazása esetén azonban nem kell számolni a HSV latenciájával, reaktivációjával és potenciális onkogenitásával kapcsolatos problémákkal. Több vacciniavírus-, adenovírus-, varicella-zoster vírus-, adeno-associated vírus- és *Salmonella typhimurium*-HSV rekombináns vakcina hatékonyságát mutatták ki állatoknál; emberi kipróbálás még nem ismert *(23–28)*.

Varicella-zoster vírus

A varicella-zoster vírus (VZV) az egyetlen olyan herpesvírus, amelynek a megelőzésére engedélyezett vakcina áll rendelkezésre. A bárányhimlő kórokozója cseppfertőzéssel, illetve hólyagbennékkel terjed. A légutakban végbemenő lokális szaporodás után a vírus a véráramba kerül, majd a bőr sejtjeit fertőzi meg, és azokban szaporodik. Retrográd axonalis transzport útján kerül az érzőganglionok neuronjaiba, ahol latens fertőzést alakít ki. A primer varicella ép immunrendszerű gyermek esetén általában jóindulatú betegség, de felnőtteknél, újszülötteknél és csökkent immunfunkciójú egyéneknél súlyos komplikációk léphetnek fel. A celluláris immunrendszer fontos szerepet játszik a vírusszaporodás visszaszorításában *(29)*. Csökkent celluláris immunválasz következtében a vírusszaporodás elhúzódó; gyakoriak a szövődményes kórformák, így a pneumonia, a máj és a központi idegrendszer fertőződése, valamint haemorrhagiás varicella. A leginkább veszélyeztetettek a leukaemiás gyermekek és a veleszületett immunhiányos állapotban szenvedők *(30)*.

A celluláris immunválasz idős korban vagy betegség, immunszuppresszív terápia esetén bekövetkező csökkenését teszik felelőssé az ezen embercsoportoknál megfigyelt herpes zoster (övsömör) kialakulásáért, amely a VZV reaktivációját jelenti *(31)*. Fontos tehát a populáció varicellaspecifikus celluláris immunválaszának fenntartása, noha bizonytalan, hogy a restimuláció elsősorban szubklinikus reaktivációk vagy a vírussal való újrafertőződések eredménye-e *(32, 33)*. Ezen tényezők ismerete fontos az esetleges tömeges vakcináció hatásának és annak megítélése szempontjából, hogy szükséges-e szeropozitív, VZV-hordozók vakcinálása.

Az Oka vírustörzset tartalmazó vakcinát, amelyben élő, attenuált vírus található, Japánban fejlesztették ki. A vírustörzset az 1970-es évek elején egy Oka nevű varicellás gyermekből izolálták; tengerimalac eredetű és humán embrionális, valamint humán diploid (WI-38) sejtekben véghezvitt sorozatos passzálással attenuálták *(34)*. A varicellavakcinát azóta számos országban, köztük Dél-Koreában, az USA-ban és Európa több országában, így Magyarországon is engedélyezték. A vakcinatörzs gyakorlatilag minden vírusantigént kifejez; antiviralis ellenanyagokat, lymphocytaproliferációs választ, citotoxikus T-lymphocytákat (CTL) egyaránt indukál *(35)*. Egészséges, egy alkalommal vakcinált gyermekeknél a szerokonverzió 97%-os, hosszú távon 85–90%-uk védett a varicellamegbetegedéssel szemben *(36, 37)*. Mindezek ellenére előfordul a fertőzésveszélynek gyakran kitett, vakcinált iskolás gyermekek között vad típusú, VZV által okozott megbetegedés, de a posztvakcinációs varicella ritka és szerencsére szinte minden esetben enyhébb lefolyású, mint a természetes fertőzés. A vakcinavírus képes latens fertőzés kialakítására, reaktivációra, és zostert okozhat. Azonban a rendelkezésre álló adatok szerint leukaemiás gyermekeknél vakcinációt követően lényegesen ritkábban fordult elő zoster, mint természetes fertőzés után, és a betegség lefolyása is enyhébbé vált *(36)*. Normális immunrendszerű gyermekeknél a vakcinációt követően ritka a kiütés, ezért feltételezhető, hogy reaktiváció is ritkán fog előfordulni *(38)*.

A Magyarországon Varilrix néven forgalomban lévő vakcina alkalmazása kilenc hónaposnál idősebb, az USA-ban forgalomban lévő vakcina (Varivax) beadása pedig 12–18 hónapos kor között javasolt. 13 éves kor fölött két vakcinadózis adása ajánlott. Hazánkban a varicellavakcina az általános vakcinációs programba még nem került be, csak magas rizikócsoportba tartozó (leukaemiás, immunszupprimált, szervtranszplantációra váró, krónikus betegségben szenvedő, veszélyeztetettek környezetében élő) gyermekek oltását engedélyezték *(39)*. Immunkompetens egyéneknél az Oka vakcina biztonságos; leggyakoribb mellékhatás az oltás helyére lokalizálódó diszkomfort, láz, kiütés az oltás helyén, esetleg testszerte enyhe, varicellához hasonló eruptió. A védőoltás kontraindikált vakcinakomponensekkel (zselatin, neomycin) szembeni túlérzékenység, kezeletlen aktív tuberkulózis és terhesség esetén. Bizonyos esetekben a vakcina alkalmazása fokozott elővigyázatosságot, körültekintést és egyedi elbírálást igényel. Fontos a beteg általános állapotának, immunstatusának mérlegelése és a folyamatban lévő gyógyszeres terápia hatásainak és mellékhatásainak monitorozása. Ellenjavalltá válhat a vakcina beadása malignus betegségek, primer vagy szerzett immunhiányos állapotok (például AIDS-szel együtt járó immunszupprimált állapot) esetén, celluláris immundeficientia, hypogammaglobulinaemia és dysgammaglobulinaemia

fennállásakor. Immunszuppresszív dózisban adott, szisztémás szteroidkezelés alkalmazásakor a vakcina csak a terápia minimum három hónapos szüneteltetése után adható be. Vér, plazma vagy immunglobulinok adása után a vakcina beadásával legalább öt hónapot kell várni *(40)*.

A tömeges vakcináció egyik fontos célja a populációban cirkuláló vad varicellavírus előfordulásának csökkentése lenne, egyrészt a védőoltásban nem részesültek, másrészt a csökkent immunfunkciójú egyének védelmében. A tömeges vakcináció kérdése azonban nem egyértelmű. A vakcina indukálta immunválasz csökkenésével a vakcináltak felnőttkorban válhatnak fogékonnyá az idősebb korban jellemző, súlyos lefolyású varicellával szemben. A populáció immunkészültségének fenntartása érdekében ezért indokolttá válhat egy második oltás alkalmazása. A vírusspecifikus immunválasz időskori vakcinációval létrehozott ismételt stimulálásával csökkenthető a zosteres esetek száma *(41, 42)*. Felmerülhet a varicellafertőzésen korábban át nem esett felnőttek védőoltásának szükségessége is, a természetes fertőzés kapcsán ebben az életkorban megfigyelt komplikációk kivédése érdekében. A klinikai tünetek kivédésére az Oka vakcina esetleges posztexpozíciós alkalmazását is javasolják *(43)*.

Cytomegalovirus

A cytomegalovirus-fertőzés közvetlen kontaktussal, nyállal, szexuális úton, vértranszfúzióval, szervtranszplantációval vagy transplacentaris úton terjed. A szülőcsatornán való áthaladás közben is fertőződhet az újszülött. A populáció átfertőzöttsége igen nagy, felnőttkorra az emberek 50–100%-a átesik az infekción. Ennek klinikai következménye a gazdaszervezet immunstatusától függ. Egészséges immunrendszerű gyermekeknél és felnőtteknél a fertőzés általában tünetmentes formában zajlik, illetve fiatal felnőtteknél mononucleosis infectiosa szindróma jön létre. Súlyos következményekkel jár a cytomegalovirus-fertőzés újszülötteknél, immunhiányos állapot esetén és transzplantációt követően. A vírus a primer fertőzés után gyakorlatilag minden esetben latenciát alakít ki a gazdaszervezetben. Az utóbbi időben gyűlnek az adatok a CMV-fertőzésnek az atherosclerosis és az angioplasticus műtétek utáni restenosis kialakulásában játszott etiológiai szerepére vonatkozóan is *(44–46)*.

A humán cytomegalovirus jelenleg a leggyakoribb congenitalis fejlődési rendellenességet okozó fertőző ágens. Az újszülöttek 0,5–2,5%-a születik intrauterin korban lezajlott HCMV-fertőzéssel; a fertőzés következményei születéskor az érintett gyermekek körülbelül 10–15%-ánál figyelhetők meg. A fertőzés a legsúlyosabb esetekben a magzat méhen belüli elhalásához vezet. A születés után súlyos tünetekkel jelentkező cytomegaliás zárványbetegség az érintett gyermekek körülbelül 5%-ánál fordul elő. Ugyancsak 5%-ban figyelhető meg atípusos megbetegedés a központi idegrendszer érintettségével. Az esetek zömében (a fertőzött gyermekek 90%-ánál) az infekció tünetei közvetlenül a születés után nem láthatók, de ezen gyermekek 10–15%-ánál (azaz az összes élve született gyermek körülbelül egy ezrelékénél) négy-öt éves korra késői következmények – mentális retardáció, chorioretinitis, microcephalia és halláskárosodás – alakulhatnak ki *(47, 48)*.

A csontvelő-, illetve szervtranszplantációt követő CMV-infekcióból eredő szövődmények az érintettek 20–60%-ánál jelentkeznek (pneumonia, hepatitis, nephritis, encephalitis, csontvelő-depresszió, bakteriális és gombás fertőzések iránti esendőség formájában) *(49, 50)*. Humán immundeficientia vírus- (HIV-) fertőzést követően az esetek 20%-ában figyelhető meg súlyos, cytomegalovirus okozta betegség.

Komoly következményekkel járó intrauterin fertőzések általában olyan anyák gyermekeinél figyelhetők meg, akiknél a terhesség alatt primer CMV-fertőzés zajlott le. Szeropozitív terhes anyák egy részénél a CMV reaktiválódik a terhesség alatt, a vírus áthatol a placentán és megfertőzi a magzatot; tartós vírusürítés is létrejöhet, de ilyen esetekben ritka a szervi károsodás *(48, 51)*. Hasonlóképpen fontos tényező a donor és a recipiens CMV-szerostatusa a transzplantációt követően kialakuló betegség szempontjából: súlyos, gyakran életveszélyes megbetegedés szeropozitív donor és szeronegatív recipiens esetében gyakori, míg szeropozitív recipiensnél a tünetek általában enyhék *(49)*. Ezek a megfigyelések arra utalnak, hogy a vírusspecifikus immunitás védelmet jelent a CMV okozta megbetegedéssel szemben, a természetes immunitás azonban nem védi meg sem a szeropozitív AIDS-betegeket, sem az egészséges szeropozitív egyéneket az újrafertőződéstől vagy a reaktivációtól *(52–55)*. A vírus ki tud térni az immunfelügyelet alól, és a primer infekciót követően krónikus vagy latens vírusfertőzést alakít ki. A reaktivációval járó vírusürítés lehetővé teszi új gazdaszervezetek fertőződését.

A szeropozitivitás CMV-specifikus celluláris immunitással is együtt jár; nem tisztázott azonban, hogy a CMV okozta betegségekkel szembeni védelemért milyen arányban felelős a humorális és milyen arányban a celluláris immunitás. A celluláris

from the donor. *N Engl J Med 1995;333:1038-44.*
60. *Gyulai Z, Endrész V, Burián K, Pincus S, Toldy J, Cox WI, et al.* Cytotoxic T lymphocyte responses to various HCMV proteins in healthy seropositive individuals: reevaluation of importance of IE-specific CTL. *J Infect Dis 2000;181:1537-46.*
61. *Elek SD, Stern H.* Development of a vaccine against mental retardation caused by cytomegalovirus infection in utero. *Lancet 1974;1:1-5.*
62. *Neff BJ, Weibel RE, Buynak EB, McLean AA, Hilleman MR.* Clinical and laboratory studies of live cytomegalovirus vaccine Ad-169. *PSEBM 1979;160:32-7.*
63. *Plotkin SA, Furukawa T, Zygraich N, Huygelen C.* Candidate cytomegalovirus strain for human vaccination. *Infect Immun 1975;12:521-7.*
64. *Marshall GS, Plotkin SA.* Progress toward developing a cytomegalovirus vaccine. *Infect Dis Clin North Am 1990;4:283-98.*
65. *Quinnan GV Jr, Delery M, Rook AH, Frederick WR, Epstein JS, Manischewitz JF, et al.* Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus. *Ann Intern Med 1984;101:478-83.*
66. *Adler SP, Hempfling SH, Starr SE, Plotkin SA, Riddel S.* Safety and immunogenicity of the Towne strain cytomegalovirus vaccine. *Pediat Infect Dis J 1998;17:200-206.*
67. *Plotkin SA, Smiley ML, Friedman HM, Starr SE, Fleisher GR, Wlodaver C, et al.* Towne-vaccine-induced prevention of cytomegalovirus disease after renal transplants. *Lancet 1984;1:528-30.*
68. *Plotkin SA, Higgins R, Kurtz JB.* Multicenter trial of Towne strain attenuated virus vaccine in seronegative renal transplant recipients. *Transplantation 1994;58:1176-8.*
69. *Plotkin SA, Huang ES.* Cytomegalovirus vaccine virus (Towne strain) does not induce latency. *J Infect Dis 1985;152:395-7.*
70. *Plotkin SA, Starr SE, Friedman HM, Gönczöl E, Weibel RE.* Protective effects of Towne cytomegalovirus vaccine against low-passage cytomegalovirus administered as a challenge. *J Infect Dis 1989;159:860-5.*
71. *Adler SP, Starr SE, Plotkin SA, Hempfling SH, Buis J, Manning ML, et al.* Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age. *J Infect Dis 1995;171:26-32.*
72. *Gönczöl E, deTaisne C, Hirka G, Berencsi K, Lin W, Paoletti E, et al.* High expression of human cytomegalovirus (HCMV)-gB protein in cells infected with a vaccinia-gB recombinant: the importance of the gB protein in HCMV immunity. *Vaccine 1991;9:631-7.*
73. *Spaete RR.* A recombinant subunit vaccine approach to HCMV vaccine development. *Transplant Proc 1991;23:90-6.*
74. *Pass RF, Duliege AM, Boppana S, Sekulovich R, Percell S, Britt W, et al.* A subunit cytomegalovirus vaccine based on recombinant envelope glycoprotein B and a new adjuvant. *J Infect Dis 1999;180:970-5.*
75. *Frey SE, Harrison C, Pass RF, Yang E, Boken D, Sekulovich RE, et al.* Effects of antigen dose and immunization regimens on antibody responses to a cytomegalovirus glycoprotein B subunit vaccine. *J Infect Dis 1999;180:1700-3.*
76. *Endrész V, Kari L, Berencsi K, Kari C, Gyulai Z, Jeney C, et al.* 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;17:50-8.*
77. *Pande H, Campo K, Tanamachi B, Forman SJ, Zaia JA.* Direct DNA immunization of mice with plasmid DNA encoding the tegument protein pp65 (ppUL83) of human cytomegalovirus induces high levels of circulating antibody to the encoded protein. *Scand J Infect Dis 1995;(Suppl)99:117-20.*
78. *Wang R, Doolan DL, Le TP, Hedstrom RC, Connan KM.* Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science 1998;282:476-9.*
79. *Boyer JD, Cohen AD, Vogt S, Schumann K, Nath B, Ahn L, et al.* Vaccination of seronegative volunteers with a Human Immundeficiency Virus type 2 env/rev DNA vaccine induces antigen-specific proliferation and lymphocyte production of β-chemokines. *J Inf Dis 2000;181:476-83.*
80. *MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, Bagarazzi ML, et al.* First human trial of DNA-based vaccine for the treatment of human immundeficiency virus type 1 infection: safety and host responses. *J Infect Dis 1998;178:92-100.*
81. *Marshall GS, Ricciardi RP, Rando RF, Puck J, Ge RW, Plotkin SA, et al.* An adenovirus recombinant that expresses the human cytomegalovirus major envelope glycoprotein and induces neutralizing antibodies. *J Inf Dis 1990;162:1177-81.*
82. *Gönczöl E, deTaisne C, Hirka G, Berencsi K, Lin W, Paoletti E, et al.* High expression of human cytomegalovirus (HCMV) -gB protein in cells infected with a vaccinia-gB recombinant; the importance of the gB protein in HCMV-immunity. *Vaccine 1991;9:631-7.*
83. *Gönczöl E, Berencsi K, Pincus S, Endrész V, Meric C, Paoletti E, et al.* Preclinical evaluation of an ALVAC (canarypox) -human cytomegalovirus glycoprotein B vaccine candidate. *Vaccine 1995;13:1080-5.*
84. *Berencsi K, Rando R, deTaisne C, Paoletti E, Plotkin SA, Gönczöl E.* Murine cytotoxic T cell response specific for human cytomegalovirus glycoprotein B (gB) induced by adenovirus and vaccinia virus recombinants expressing gB. *J Gen Virol 1993;74:2507-12.*
85. *Berencsi K, Gönczöl E, Endrész V, Kough J, Takeda S, Gyulai Z, et al.* 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 (UL123) induce a cytotoxic T-cell response. *Vaccine 1996;14:369-74.*
86. *Magno A, Smith GL, Moss B.* Vaccinia virus: a selectable eukaryotic cloning and expression vector. *PNAS USA 1982;79:7415-9.*
87. *Lane JM, Ruben FL, Neff JM, Millar JD.* Complications of smallpox vaccination, 1968: National surveillance in the United States. *N Engl J Med 1969;281:1201-8.*
88. *Taylor J, Meignier B, Tartaglia J, Languet B, VanderHoeven J, Franchini G, et al.* Biological and immunogenic properties of a canarypox-rabies recombinant, ALVAC-RG (vCP65) in non avian species. *Vaccine 1995;13:539-49.*
89. *Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI, et al.* NYVAC: a highly attenuated strain of vaccinia virus. *Virology 1992;188:217-32.*
90. *Plotkin SA, Cadoz M, Meignier B, Meric C, Leroy O, Excler JL, et al.* The safety and use of canarypox vectored vaccines. *Dev Biol Stand 1995;84:165-70.*
91. *Adler SP, Plotkin SA, Gönczöl E, Cadoz M, Meric C, Wang JB, 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.*
92. *Gyulai Zs, Pincus S, Cox WI, Meric C, Cadoz M, Zahradnik J, et al.* Canarypox-CMV-pp65 recombinant immunization of seronegative subjects elicits pp65 specific CTL precursors with a frequency comparable to that of naturally seropositive individuals. 7th International Cytomegalovirus Workshop, Brighton, Great Britain. *J Clin Virol 1999;12:164.*
93. *Berencsi K, Gyulai Z, Pincus S, Cox WI, Meric C, Cadoz M, et al.* 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 Clin Virol 1999;12:165.*
94. *Miyashita EM, Yang B, Babcock GJ, Thorley-Lawson DA.* Identification of the site of Epstein-Barr virus persistence in vivo as a resting B-cell. *J Virol 1997;71:4882-91.*
95. *Khanna R, Burrows SR, Moss DJ.* Immune regulation in Epstein-Barr virus-associated diseases. *Microbiol Rev 1995;59:387-405.*
96. *Hanto DW.* Classification of Epstein-Barr virus-associated post-transplant lymphoproliferative diseases: implications for understanding their pathogenesis and developing rational treatment strategies. *Annu Rev Med 1995;46:381-94.*
97. *Morgan AJ.* The development of Epstein-Barr virus vaccines. *Vaccine 1992;10:563-71.*
98. *Morgan AJ, Mackett M, Finerty S, Arrand JR, Scullion FT, Epstein MA.* Recombinant vaccinia virus expressing Epstein-Barr virus glycoprotein gp340 protects cottontop tamarins against virus-induced malignant lymphomas. *J Med Virol 1988;25:189-95.*
99. *Gu SY, Huang TM, Ruan L, Miao YH, Lu H, Chu CM, et al.* First EBV vaccine trial in humans using recombinant vaccinia virus expressing the major membrane antigen. *Dev Biol Stand 1995;84:171-7.*
100. *Morgan AJ, Finerty S, Lovgren K, Scullion FT, Morein B.* Prevention of Epstein-Barr (EB) virus-induced lymphoma in cottontop tamarins by vaccination with the EB virus envelope glycoprotein gp340 incorporated into immune-stimulating complexes. *J Gen Virol 1988;69:2093-6.*
101. *Wilson AD, Lovgren-Bengtsson K, Villacres-Ericsson M, Morein B, Morgan AJ.* The major Epstein-Barr virus (EBV) envelope glycoprotein gp340 when incorporated into Iscoms primes cytotoxic T-cell responses directed against EBV lymphoblastoid cell lines. *Vaccine 1999;17:1282-90.*
102. *Moss DJ, Schmidt C, Elliott SL, Suhrbier A, Burrows SR, Khanna R.* Strategies involved in developing an effective vaccine for EBV-associated diseases. *Adv Cancer Res 1996;69:213-44.*

Literatura Medica Kiadó 1539 Budapest, Pf. 603; E-mail: litmed@elender.hu
LAM egyéni előfizetési díj egy évre: 3864 Ft, ZÖLD SZÁM: 06 80 20 10 22