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CMV vaccine development based on epithelial entry mediators UL128, UL130, UL131

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CMV VACCINE DEVELOPMENT BASED ON EPITHELIAL ENTRY MEDIATORS UL128, UL130, AND UL131

A Dissertation submitted in partial fulfillment of the requirements for the degrees of Doctor of Medicine and Doctor of Philosophy at Virginia Commonwealth University

By

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LIST OF ABBREVIATIONS

Ad5	Adenovirus type 5 vector
AIDS	Acquired immune-deficiency syndrome
ALVAC	Canarypox vaccine vector
Bad*r*UL131	CMV strain AD169 with repaired UL131 gene
BALT	Bronchus-associated lymphoid tissue
BSA	Bovine serum albumin
CHO	Chinese hamster ovary cells
CDV	Cidofovir
CID	Cytomegalic inclusion disease
CMIS	Common mucosal immune system
CMV	Human cytomegalovirus
CNS	Central nervous system
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
D/R arm	Donor/recipient pairs of VCL-CB01 phase II trial
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FOS	Foscarnet
GALT	Gut-associated lymphoid tissue
GPCMV	Guinea pig cytomegalovirus
GCV	Ganciclovir
HBcAg	Hepatitis B core antigen
HIV	Human immune-deficiency virus
HSCT	Hematopietic stem cell transplant
IFN	Interferon
KLH	Keyhole limpet hemocyanin
KSFM	Keratinocyte serum-free media
LPS	Lipopolysaccharide
MALT	Mucosal-associated lymphoid tissue
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
M cells	Microfold epithelial cells
MEM	Modified Eagle medium
MVA	Modified vaccinia Ankara
NALT	Nasopharynx-associated lymphoid tissue

NK cells	Natural killer cells
O.D.	Optical density
ORF	Open reading frame
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% Tween-20
PFU	Plaque forming units
pIgA	Polymeric IgA
pIgR	Polymeric Ig receptor
PKR	Protein kinase R
PRR	Pattern-recognition receptors
R arm	CMV seropositive recipient only arm of VCL-CB01 phase II trial
RhCMV	Rhesus macaque CMV
rMVA	Recombinant modified vaccinia Ankara
RPM	Revolutions per minute
rVSV	Recombinant vesicular stomatisis virus
sIgA	Secretory IgA
SNHL	Sensorineural hearing loss
SOT	Solid organ transplant
TLR	Toll-like receptor
UL	Unique long
US	Unique short
VLP	Virus-like particle

ABSTRACT

CMV VACCINE DEVELOPMENT BASED ON EPITHELIAL ENTRY MEDIATORS UL128, UL130, AND UL131

By Frances Maria Saccoccio

A Dissertation submitted in partial fulfillment of the requirements for the degrees of Doctor of Medicine and Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2011

Major Director: Michael A. McVoy
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Congenital cytomegalovirus infection is the leading cause of sensorineural hearing loss in the U.S. CMV vaccines developed to date do not protect the majority of women of childbearing age from primary CMV infection. Insufficient vaccine-induced epithelial entry neutralizing activity may be the reason for poor performance of these vaccines. CMV entry into endothelial and epithelial but not fibroblast cells requires the virion envelope complex gH/gL/UL128-131. Since current vaccines do not target this complex, epithelial entry mediators UL128-131 are attractive subunit CMV vaccine candidates, since they should target mucosal immunity. The mucosal immune response, specifically salivary epithelial entry neutralizing activity, has not been previously described. This report demonstrates that salivas from CMV seropositive children under two, adolescents, and Towne vaccine recipients do not have epithelial or

fibroblast neutralizing activity. Epithelial but not fibroblast neutralizing activity was identified in half of the salivas from CMV seropositive adults tested. This activity correlated with the level of serum neutralizing activity, suggesting that salivary neutralizing activity results from passively transferred serum IgG. Furthermore, this report describes three highly immune individuals with serum and saliva neutralizing titers two- to four-fold above average. These individuals also have UL130 antibodies detectable in western blot assays. This is the first report of antibodies by western blot in CMV seropositive sera to UL128, UL130, or UL131. To determine the feasibility of UL128-131 as vaccine candidates both peptide and DNA vaccines were tested in animal models. Rabbit anti-peptide sera from UL130 and UL131 vaccinated animals induced epithelial entry neutralizing activity similar to that found following natural infection. Mixing anti-peptide UL130 and UL131 sera neutralized CMV infection of epithelial cells at titers higher than natural infection. DNA vaccination with these proteins was not as successful but based on DNA vaccination of mice UL130 is the most immunogenic of the three proteins. These data support further development of UL130 as a CMV vaccine. Future vaccines, including the vaccine candidates described in this report, should strive to induce levels of immunity seen in the three highly immune individuals, specifically serum epithelial neutralizing titers >1:7,000 and saliva epithelial neutralizing titers >1:20.

INTRODUCTION

Cytomegalovirus

Cytomegalovirus (CMV) is the prototypical betaherpesvirus, also known as human herpesvirus 5. Similar to other herpesviruses, CMV is species specific with CMVs identified for most mammalian species including human, rhesus macaque, mouse, rat, and guinea pig. Cytomegaloviruses are ubiquitous in the animal kingdom but cause different disease syndromes in various species. For example, human CMV can cross the placenta and cause devastating congenital infections while murine cytomegalovirus (MCMV) does not infect the fetus. Due to significant differences between species-specific viruses, small animal models are not ideal for vaccine development. Cytomegaloviruses have been evolving with mammals for more than ten million years and have impacted the genome of its hosts. For example, more than three percent of mouse genome is dedicated to inhibiting MCMV infection.[1]

Impact on human health

Approximately half of the population of the U.S. will become infected with CMV by the age of 50.[2] The transmission rate of CMV to seronegative individuals is about 1-2% per year.[3] Adolescents have a higher attack rate than the general population, approximately 13%.[4] The virus is shed in saliva, urine, semen, cervical secretions and breast milk. CMV can also be

transmitted by blood transfusion, organ transplant, and sexual contact. The vast majority of these infections are asymptomatic with minimal impact on the infected individual. CMV infection becomes latent and can reactivate if an individual becomes immune compromised later in life. Significant CMV disease occurs in immune-compromised individuals (solid organ transplant and AIDS patients) and neonates infected *in utero*.[3] Reactivation of CMV infection can also occur in healthy adults but this leads to subclinical disease. T cell expansion due to subclinical reactivated CMV has been hypothesized to drive immune senescence in the elderly.[5]

Congenital disease

Congenital infection affects about 40,000 neonates per year in the U.S., leading to about 200 deaths yearly and up to 8,000 cases of permanent neurologic disability.[2,6-8] The vast majority (80-90%) of these neonates are asymptomatic at birth but about 10-15% will develop neurologic disabilities over time. In contrast, symptomatic infants develop cytomegalic inclusion disease (CID) which is associated with intra-uterine growth retardation, jaundice, hepatosplenomegaly, microencephaly, and thrombocytopenia.[9] In addition, approximately 50% of children with CID will develop neurologic disability.[7] Anti-viral treatments are available for symptomatic infants but these treatments frequently cause neutropenia and have the potential to cause infertility in males. Treatment reduces hearing loss and developmental delay in mildly symptomatic infants but anti-virals have a minimal effect on the overall outcome of severely symptomatic infants.[10]

CID occurs more frequently when a woman develops a primary CMV infection during pregnancy. If acquired during pregnancy, the transmission rate is about 50%. In contrast, the transmission rate when infection occurs prior to pregnancy is 0.5%-2%.[3] These statics translate to a three-fold higher risk of CMV transmission to the fetus in CMV seronegative women.[4] A

major risk factor for CID is the presence in the household of children in daycare. Young children shed CMV in their urine and saliva for up to 18 months.[3] Hand washing and reducing exposure to urine and saliva from young children is key to preventing CMV acquisition during pregnancy.[8] Additional risk factors for development of congenital CMV include maternal age under 25 years and short spacing between pregnancies.[4]

Pre-conceptional maternal immunity to CMV reduces the incidence and severity of congenital CMV disease.[3] In cases of primary CMV infection during pregnancy, incidence and severity of CMV disease is related to the nature of the antibody response developed during pregnancy. Women that transmit CMV to their fetus have lower fibroblast entry neutralizing antibody titers but higher CMV IgG and anti-gB antibodies than women that do not transmit CMV to their fetus. The avidity of antibodies in nontransmitters is higher than that of transmitters ($p<0.002$). The presence of high avidity antibodies also correlates with the level of neutralizing antibodies.[11] Timing of primary CMV infection during pregnancy influences transmission to the fetus. Relative transmission rates based on the trimester when primary CMV infection occurs are 50% in the first trimester, 40% in the second trimester, and 71% in the third trimester.[12,13] Eventhough transmission during the first trimester occurs at a lower frequency than transmission during the third trimester, neonatal disease is more severe when transmission occurs in the first trimester.[14]

CMV transmission to the fetus is limited by neutralizing antibody suppressing viral replication in the placenta. Administration of CMV IVIG to pregnant women with primary CMV infection reduces transmission and the severity of disease in the fetus.[15] High avidity IgG1 antibodies in the maternal serum have been implicated in reduction of viral replication.[16] Transmission of CMV to the fetus in women with a history of CMV immunity before pregnancy

is rare but can result either from CMV reactivation or from the acquisition of a new CMV strain during pregnancy. CMV seropositive women that acquire a new CMV strain during pregnancy are more likely to transmit CMV to their fetus (17.5%) than seropositive women that do not acquire a new CMV strain during pregnancy (4.6%). A higher percentage of congenitally infected infants were born to women infected with multiple strains of CMV (52.5%) than women infected with only a single strain of CMV (20%).[17] Recently, a case report was described of a woman that transmitted the same CMV strain to two fetuses approximately two years apart. The woman described in this case had no identifiable immune system dysfunction. This case supports the rare occurrence of reactivated CMV causing congenital infection.[18]

Pathology

CMV crosses the placenta and infects the central nervous system (CNS) of a developing fetus. A variety of cell types in the CNS support CMV infection including astrocytes, microvascular endothelial cells, microglia, macrophages, oligodendrocytes, and neurons. Progenitor cells in the developing CNS of the fetus are more susceptible to CMV infection than the differentiated CNS of a adult, which supports the clinical observation that immune-compromised adults rarely suffer from CNS infections while neonates frequently suffer neurologic damage.[9]

Sensorineural hearing loss (SNHL) is the most common neurologic sequalae of CID. CMV infection of fetal CNS tissue alters the development of inner ear structures and vestibular organs. CNS damage is related to viral load with neonates that shed CMV at levels less than 5,000 PFU/ml in their urine not likely to develop hearing loss overtime. Progressive hearing loss

seen in CID is most likely due to chronic CMV infection of the CNS since children with progressive SNHL shed CMV in their urine for at least four years.[9]

Incidence of neurologic sequelae

Approximately 17-20% of infected infants will develop permanent neurologic sequelae with the majority of disease incidence coming from children asymptomatic at birth (Fig 1). Assuming 1000 infants born with congenital CMV infection, 127 (12.7%) will show symptoms of CID while 873 (87.3%) will be asymptomatic. Of the 127 symptomatic neonates, five will die (0.5%) while 122 will survive. No asymptomatic children will die. 50-70 (40-58%) of symptomatic neonates will develop permanent neurologic sequelae, such as SNHL and cognitive difficulties. In contrast 118 (13.5%) asymptomatic neonates will develop sequalae; mostly SNHL. Due to the large size of the asymptomatic group (873 neonates vs 127 neonates), 2/3 of children born with congenital CMV infection that develop permanent neurologic sequelae are asymptomatic at birth.[7]

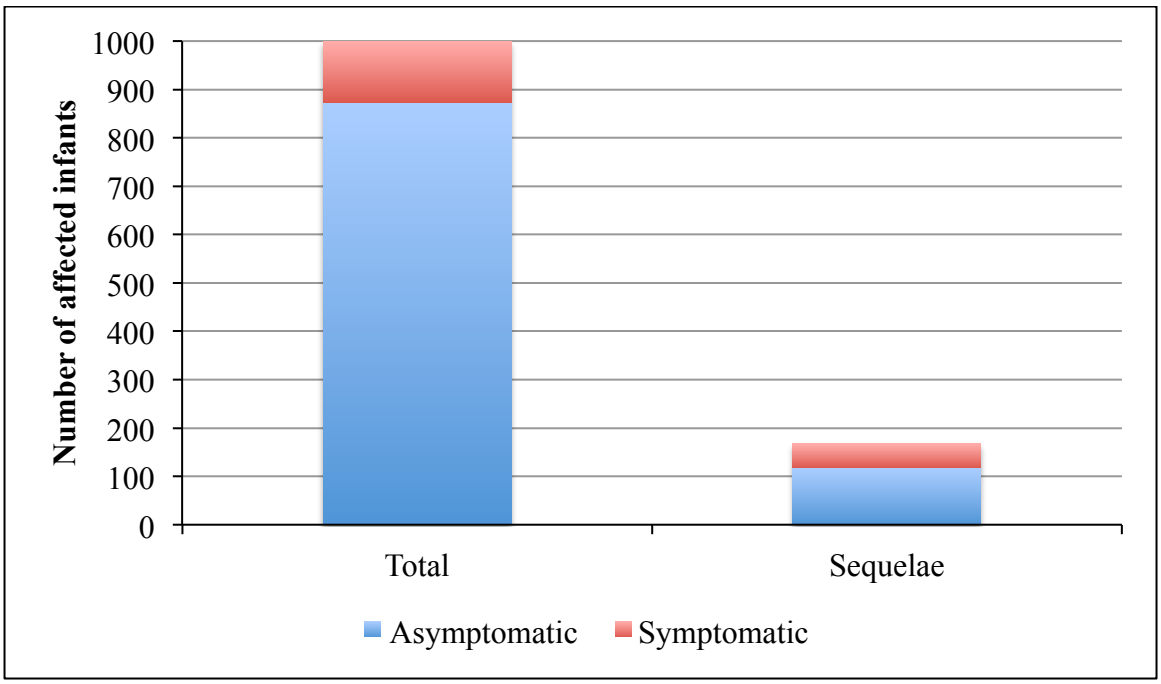


Figure 1: The majority of neonates that develop sequelae from congenital CMV infection are asymptomatic at birth. Approximately two thirds of cases of neurologic sequalae following congenitally infection occur in children asymptomatic at birth.[7]

Immune compromised populations

About 50% of adults in the U.S. are seropositive and hence latently infected by CMV.[2] Reactivation from latency occurs upon immune suppression so CMV causes disease in immune-compromised individuals, such as solid organ transplant (SOT), hematopoietic stem cell transplant (HSCT) recipients, and individuals with human immune-deficiency virus/acquired immune-deficiency syndrome (HIV/AIDS). Disease in this population includes symptoms such as hepatitis, pneumonia, lymphadenopathy, enteritis and retinitis. Severity of CMV disease in immune-compromised individuals correlates with the level of immune suppression.[3]

HIV/AIDS

CMV causes disease in about 40% of HIV/AIDS patients not receiving effective anti-retroviral treatment. The majority of disease in this population occurs as retinitis.[5] AIDS patients with advanced immune suppression also suffer from CMV-associated pneumonia, enteritis, and rarely encephalopathy.[3]

Transplant

CMV disease can either reactivate from latency in the transplant recipient, develop from the transplanted organ regardless of the recipient sero status, or be acquired from blood products given during surgery. Following SOT of an organ from a seropositive donor, CMV will reactivate in at least 90% of the cases. CMV reactivation increases the likeliness of organ rejection.[3] Severe disease is most common when the recipient is CMV seronegative and receives organs, tissues, or blood products from seropositive donors. SOT patients develop CMV syndrome, which is characterized by fever, malaise, leukopenia, rash, and organ-specific disease. Seropositive HSCT patients suffer disease from CMV reactivation approximately 80% of the

time. In contrast only about 30% of seronegative HSCT recipients will develop a primary CMV infection. Pneumonitis and enteritis are the most common types of CMV disease in this population.[5]

Structure and life cycle

CMV, like other herpes viruses, is an enveloped double-stranded DNA virus. The CMV genome is the largest of the human herpes viruses at 235 kb with a predicted 165 open reading frames (ORFs). The genome is divided into two regions, the unique long (UL) and unique short (US) regions. An icosahedral capsid surrounds the genome. A layer of tegument proteins exists between the capsid and envelope. The majority of viral proteins are in this tegument layer with pp65 being the most abundant protein. The host-derived lipid bilayer envelope contains at least 20 CMV-encoded glycoproteins. Virion envelope glycoproteins such as gB, gH, gL, gM, gN, and gO are involved in attachment and entry of the virus into host cells.[5]

Prior to viral replication, CMV must transport its DNA to the host cell nucleus (Fig 2). The initial step to this process is attachment of the virion to the host cell surface via unidentified host cell receptors (step 1 Fig 2). Following attachment the viral envelope fuses with the cellular membrane, which releases the capsid into the cytosol (step 2 Fig 2). Membrane fusion occurs in different locations in different cell types (see below and step 2 Fig 2). After arriving in the cytosol, capsids travel to the nucleus and dock at nuclear pores where they inject viral DNA (step 3 Fig 2). Once the DNA reaches the nucleus, viral gene expression occurs in three waves: immediate early, early, and late gene expression (step 4 Fig 2). Immediate early genes are transcribed within the first few hours of infection and encode viral genes that modulate virus and host gene expression. Early gene transcription requires immediate early gene-encoded proteins. The early genes encode mostly viral DNA replication proteins. Finally, late genes are

transcribed about 24 hours post infection. Transcription of these genes requires early gene-encoded proteins and DNA synthesis. Late genes encode for virion structural proteins and proteins involved in virion assembly and egress, including glycoproteins.[4]

Viral mRNAs are translated in ribosomes associated with the endoplasmic reticulum (ER) and cytosol (step 5 Fig 2) then proteins are processed in the Golgi (step 6 Fig 2). Vesicles carry viral envelope proteins from the Golgi to the plasma membrane (step 8 Fig 2). Fully processed viral envelope proteins return to the cytosol in the membranes of vesicles formed from the plasma membrane (step 9 Fig 2). Virion structural proteins are transported to the nucleus where capsids are assembled (step 10 Fig 2). Viral DNA replicates in the nucleus as concatemers. One unit length genome is packaged into a capsid and cleaved from the concatemer prior to capsid export from the nucleus (step 11 Fig 2). Capsids initially gain some tegument proteins during a primary envelopment at the inner nuclear envelope. De-envelopment at the outer nuclear membrane delivers the capsid to the cytosol. Capsids then migrate to Golgi-derived vesicles where they acquire the remaining tegument proteins. Capsids bud into Golgi-derived vesicles for final envelopment. The mature virions are released from the cell via exocytosis (step 12 Fig 2).[19]

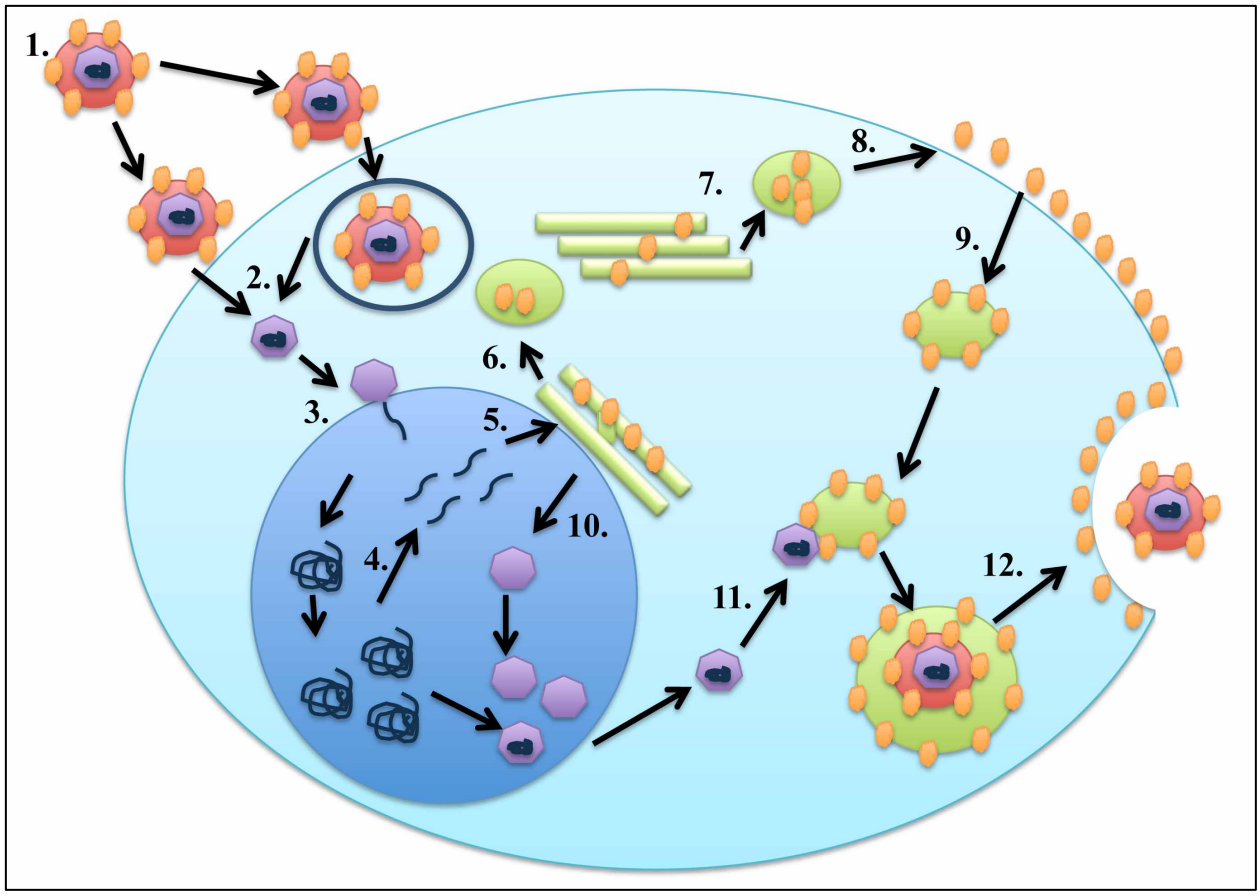


Figure 2: CMV life cycle. The CMV virion contains double-stranded DNA (dark blue) enclosed in an icosahedral capsid (light purple) surrounded by a lipid envelop (red) with embedded glycoproteins (orange). The virus attaches to cell surface receptors (step 1) prior to entry into the cell via fusion at the plasma membrane or endocytosis (step 2). Once the viral genome is released into the nucleus (light blue) (step 3), gene expression occurs in three waves: immediate early, early, and late (step 4). Viral proteins (orange) are processed in the cytosol and ER (green) (step 5) and some transit through the Golgi (green) (step 6) and are transported to the plasma membrane in Golgi derived vesicles (green) (steps 7 and 8). Vesicles bud off the plasma membrane with viral proteins in the membrane (step 9). After leaving the nucleus, the mature capsid with genome inside becomes enveloped by budding into vesicles (step 11) then leaves the cell via exocytosis (step 12).[5]

Entry pathways

Fibroblasts

CMV enters fibroblast cells via pH independent fusion at the plasma membrane[20] (Fig 3). This process requires the glycoproteins gB, and gH/gL on the virion surface.[21] Glycoprotein gO is required to express gH/gL in the virion envelope but is not required in the envelope for virus entry.[22,23] CMV quickly adapts to growth in fibroblast cells by deleting genes, such as RL13, UL128, UL130, and UL131, that impair growth and/or replication in cell culture.[24]

Epithelial and Endothelial

CMV enters epithelial and endothelial cells via endocytosis followed by low pH triggered fusion of the virion envelope with the host cell vesicle membrane (Fig 3). This entry pathway requires a different glycoprotein complex than the fibroblast entry pathway; gB, gH/gL/UL128, UL130, and UL131 (gH/gL/UL128-131).[20,25-30] All three of the UL128-131 proteins must be expressed by the virus to form a functional complex in the virion envelope.[29] This protein complex may be involved in membrane fusion[31] and/or entry receptor recognition.[28] Interestingly, fibroblast adaptation of CMV quickly results in mutations in the UL128-131 locus creating viruses unable to enter epithelial or endothelial cells.[24] All fibroblast adapted strains of CMV lack functional genes encoding one of these three proteins, and repairing these mutations restores epithelial and endothelial entry.[25] gH/gL complexes present in the virion envelope in the absence of UL131 or UL128-UL131 most likely contain gO.[32] Virions that encode a functional UL128-131 locus express very little gO on the virion surface.[22] gO acts as a chaperone for gH/gL and appears to be replaced by UL128, UL130, and UL131 prior to surface expression.[23]

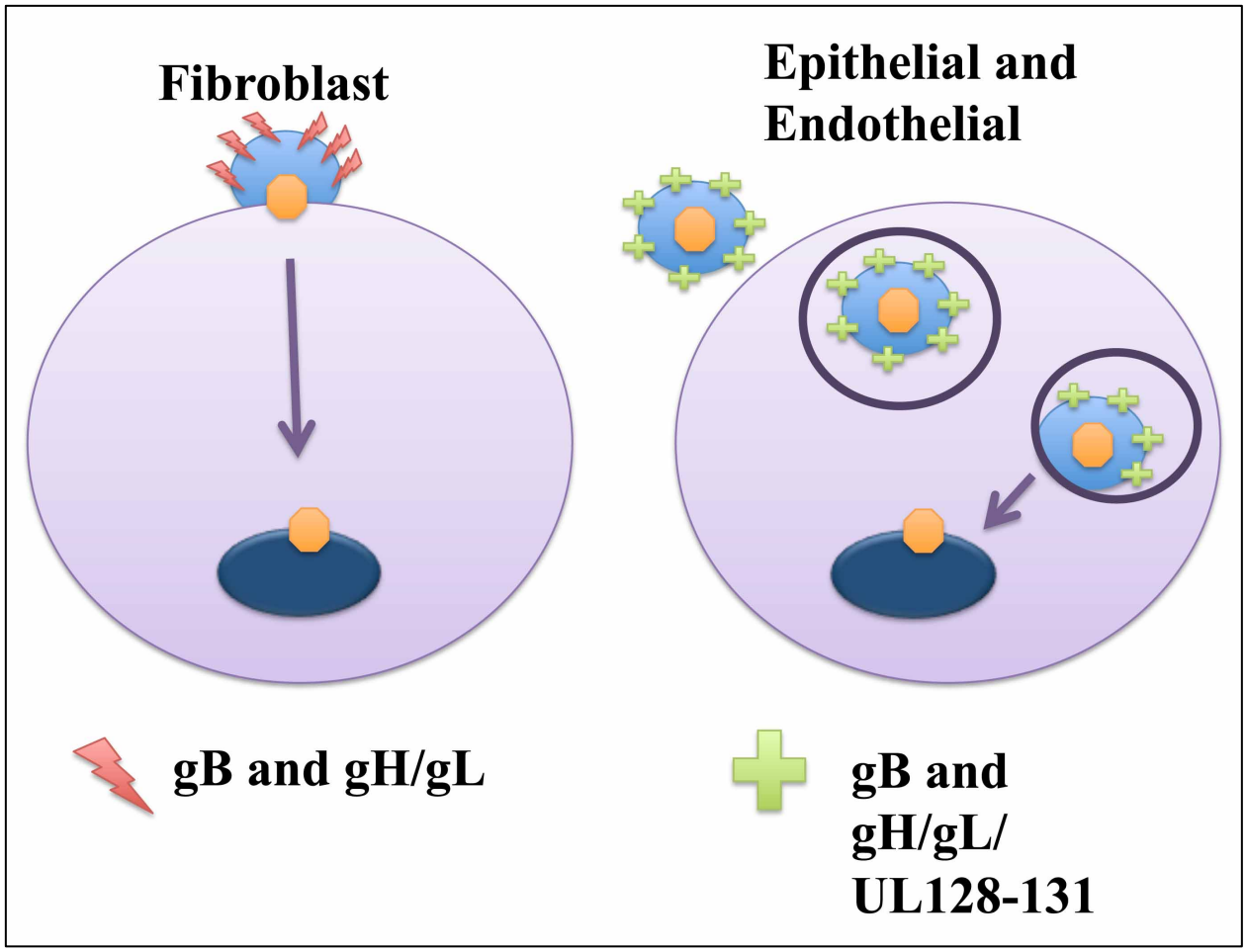


Figure 3: CMV entry pathways. CMV enters fibroblast cells via pH-independent fusion at the plasma membrane.[20] This process requires the glycoproteins gB and gH/gL in the envelope and gO expression in the host cell.[22,23] CMV enters endothelial and epithelial cells via pH-dependent endocytosis using gB and the gH/gL/UL128-131 complex.[20,29]

UL128, UL130, and UL131 are transcribed as late genes and all three proteins are predicted to be secreted.[29] UL128 has a CC-type chemokine domain and UL130 has a domain similar to CXC chemokines, but the relevance of these domains are unknown. UL128 and UL130 also share similar domains in the N-terminal signal peptide, and a unique C-terminal region.[33] Recent evidence suggests that the unique C-terminal region of UL130 contains motifs important for cell tropism.[34] UL130 is the largest of the three proteins at approximately 25 kDa. When expressed *in vitro*, the N-terminal signal sequence of UL130 is cleaved, then the protein is modified in the ER at three predicted N-linked glycosylation sites. Less than one percent of total UL130 is secreted when the protein is solely expressed. UL130 is most likely retained in a pre-Golgi compartment in the absence of UL128 and UL131.[33]

The gH/gL/UL128-131 complex structure is poorly understood. Figure 4 represents a schematic of the proposed structure of this complex. Table 1 summarizes the gH/gL/UL128-UL131 interactions. Based on immuno-precipitation experiments, when UL128, UL130, and UL131 are present UL128 binding to gH/gL is reduced by almost 50%. In the presence of UL128 the binding of UL130 to gH/gL is increased five-fold and the binding of UL131 to gH/gL increases three-fold. When UL128 is absent but UL131 is present the binding of UL130 to gH/gL is reduced. UL130 and UL131 are joined by disulfide bonds to form a dimer. Noncovalent interactions occur between gL/UL128 and gH/UL130. UL128 interacts with UL130 since anti-UL130 antibodies precipitate UL128. UL131 binds the gH/gL dimer but not the individual proteins. These data suggest that UL128, UL130, and UL131 assemble onto a gH/gL core (Fig 4).[32]

Interestingly, epithelial cells support both endocytic and non-endocytic entry of virions that contain functional UL128, UL130, and UL131 proteins. CMV strain AD169 with a repaired

UL131 gene (Bad*r*UL131) grown in fibroblast cells infects epithelial cells via low pH dependent endocytosis. In contrast, the same virus grown in epithelial cells infects epithelial cells in a manner that is less sensitive to lysosomotropic drugs but still requires UL130. Bad*r*UL131 virus grown in epithelial cells causes frequent syncytia while the same virus grown in fibroblast cells rarely causes syncytia. UL130 neutralizing antibodies block infection of epithelial cells by both fibroblast and epithelial cell-derived Bad*r*UL131 in a dose dependent manner. These differences may be due to a higher ratio of gH/gL/UL128-UL131 to gH/gL/gO in epithelial cell-derived virus compared to fibroblast-derived virus.[27]

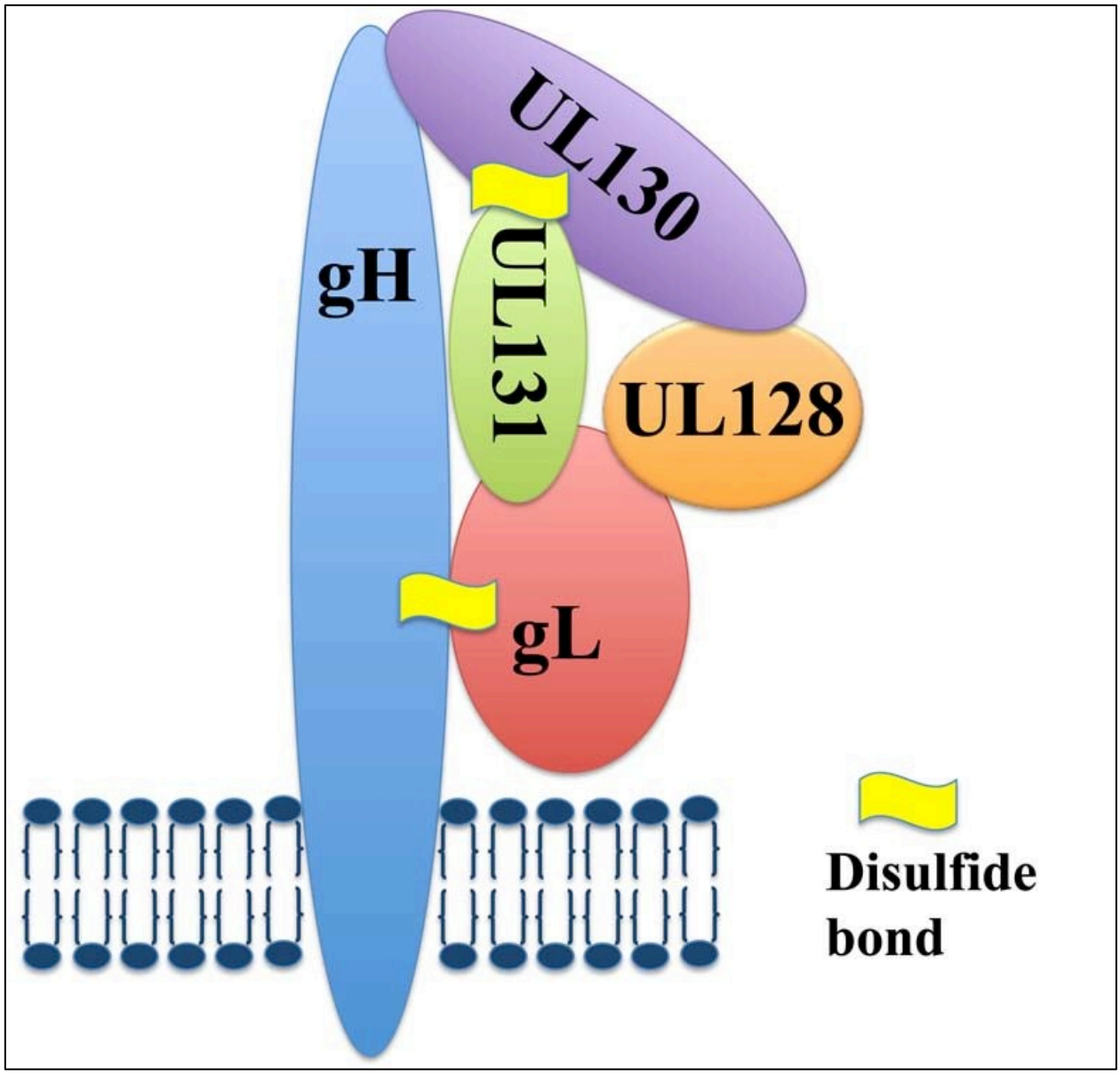


Figure 4: Schematic of the gH/gL/UL128-UL131 complex. Disulfide bonds exist between gH/gL and UL130/UL131, all other interactions are non-covanlent. gH is the only membrane anchored protein.[21,32]

Table 1: gH/gL/UL128-UL131 Interactions.[32]

Proteins Involved	Interactions
gH/gL/UL128, UL130, UL131	gH/gL/UL128 decreased gH/gL/UL130 increased gH/gL/UL131 increased
gH/gL/UL130, UL131	gH/gL/UL130 decreased
gH/gL	Disulfide bond
gH/gL/UL131	Non-covalent bond
gH/UL130	Non-covalent bond
gL/UL128	Non-covalent bond
UL128/UL130	Non-covalent bond
UL130/UL131	Disulfide bond

Anti-virals

Currently, three anti-virals are approved for control of CMV infection, all of which cause early chain termination of the viral DNA polymerase (Table 2). Ganciclovir (GCV), commonly given in its more bioavailable form Val-ganciclovir, is a guanosine analogue that requires tri-phosphorylation prior to being incorporated into a growing DNA chain where it terminates DNA replication. The viral protein kinase UL97 performs the initial phosphylation step, and then cellular kinases provide two additional phosphate groups. Foscarnet (FOS) is a pyrophosphate analogue that blocks pyrophosphate release by the viral DNA polymerase. Cidofovir (CDV) is a CMP analogue that requires phosphorylation by cellular kinases for activation. Only GCV requires a viral enzyme for activation.[35]

GCV is a teratogen so it cannot be used to treat CMV infections *in utero*. Symptomatic congenitally infected neonates respond to GCV treatment with less hearing loss over time, but the treatment causes neutropenia so its use is not ideal.[9,10] GCV therapy is effective in AIDS and transplant patients but drug resistance develops quickly, especially in the transplant population.[35] FOS and CDV are second line therapies that are not commonly used, except in cases of severe disease or GCV resistance, due to drug toxicity such a nephrotoxicity.[9]

Table 2: CMV anti-virals[9,35]

Anti-viral	Mechanism of Action	Target	Toxicity
Gancyclovir (GCV)	Guanosine analogue	Inhibits DNA Pol (UL54) Requires UL97 for activation	Neutropenia Thrombocytopenia Teratogen
Foscarnet (FOS)	Phyrophosphate analogue	DNA polymerase	Nephrotoxicity
Cidofovir (CDV)	CMP analogue	Inhibits DNA Pol (UL54)	Neutropenia Nephrotoxicity

Host defenses against CMV

The human immune system has two major branches, the innate and adaptive responses. Innate immunity is a primitive first line of defense that is generally not pathogen specific. Adaptive immunity evolves to be pathogen specific where individual cells recognize specific pathogen proteins. Adaptive immunity is comprised of cell mediated immunity (T cells) and humoral immunity (antibodies and B cells). These two arms of the immune system work together to prevent and control infections. Mucosal immunity protects the many mucosal linings of the body with an intricate interplay of both arms of the immune system.

Pathogens are recognized by the immune system based on immunogens that they express. An immunogen is a nucleic acid, lipid, protein or carbohydrate that can stimulate the immune system. Antigens represent the part of the molecule recognized by the immune system. Epitopes can be either linear or conformational and represent the molecular structure being recognized by an antibody or T cell receptor. Haptens are molecules too small to act as immunogens but become immunogenic when conjugated to an adjuvant or carrier molecule.[36]

Innate immunity

Invading pathogens initially encounter the innate immune system that consists of physical barriers (*i.e.,* skin), complement, and specialized immune cells such as macrophages, neutrophils and natural killer (NK) cells. Epithelial cells lining external surfaces secrete a variety of anti-microbial agents such as lysozyme and defensins. Once inside a host the next line of defense against pathogens is complement. The complement system can be activated by two mechanisms: the classical pathway and the alternative pathway. Innate defense makes use of the alternative complement pathway. Pathogens bind to spontaneously cleaved C3 forming the C3bBb or C3 convertase of the alternative pathway on the pathogen surface. Formation of this complex on a

pathogen surface promotes the deposition of additional complexes, which eventually target the pathogen for phagocytosis by macrophages. Complement deposition on the pathogen surface can also result in the formation of the alternative C5 convertase that forms pores in the pathogen membrane and releases C5a, which recruits neutrophils to phagocytize the invading pathogen.[36]

Pathogens express pathogen-associated molecular patterns (PAMPS) that bind to pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) bind to PAMPs, which induce pro-inflammatory cytokines and type I interferon (IFN) to induce inflammation and activate intracellular defense mechanisms. Eleven TLRs that bind a variety of PAMPs have been identified in humans. The C-terminal region of TLRs are conserved across species. TLRs are either expressed on the cell surface (TLRs 2, 4, 5, and 6) or expressed in endosomes (TLRs 3, 7, 8, and 9). Endosomal TLRs recognize viral DNA and RNA. These receptors are expressed on a wide variety of cells including DCs, endothelial cells, and epithelial cells. TLRs signal through two main pathways, MyD88-dependent and TRIF-dependent. TLRs 1, 2, 4, 5, 6, 7, and 9 signal via MyD88 to up-regulate pro-inflammatory cytokines. TLRs 7 and 9 also induce IFN-α. TLR3 signals through a TRIF pathway that induces IFN-β.[37]

Macrophages and neutrophils play an essential role in pathogen removal. Macrophages reside in tissues and provide initial pro-inflammatory signals. In contrast, neutrophils circulate in the blood and arrive at the site of pathogen infection by responding to macrophage pro-inflammatory signals. Neutrophils phagocytize pathogens coated with both complement and antibodies. Pathogens initially enter phagosomes, which quickly fuse with vesicles containing preformed granules that contain various degradative enzymes and antimicrobial enzymes forming a phagolysosome. Once inside a phagolysosome, pathogens are killed and eliminated from the cell.

NK cells also play a pivotal role in the innate immune response to viruses. NK cells are large lymphocytes with cytotoxic granules that are activated by type I IFN or activating ligands on the cell surface to kill virus-infected cells. NK cell killing is inhibited by MHC I on the surface of target cells, which is often down regulated by viral infection. Type I IFN includes IFN-α and IFN-β, and is induced by various events following viral infection or by TLR-3 activation from double-stranded RNA.[36] Most cells will release type I IFN when stressed by infection, and this primes neighboring cells in advance of infection, leading to an anti-viral state. Cellular IFN responses block viral genome replication and protein synthesis by inducing an anti-viral state through up-regulating protein kinase R (PKR) and other factors. PKR is triggered by dsRNA to shut down cellular protein synthesis by inhibiting translation initiation to prevent viral replication.[38] Type I IFN also primes infected cells for degradation by promoting recognition by NK cells and CD8 T cells. Type I IFN stimulates NK cell proliferation and maturation.[36]

Cell mediated immunity

T cells provide cell-mediated immunity, which is exquisitely pathogen specific. The T cell receptor recognizes small peptides from degraded pathogen proteins loaded onto major histocompatibility complexes (MHC). T cells can either kill targeted cells (CD8 T cells) or stimulate B cells to make antibodies (CD4 T cells). Dendritic cells (DCs) monitor the body for pathogens and migrate to lymphoid tissue to present peptides from potential pathogens on MHCs to T cells. A naïve T cell that encounters dendritic cells expressing a peptide recognized by its T cell receptor becomes activated and will proliferate and differentiate.[36]

CD4 cells

CD4 T cells, also known as helper T cells, recognize peptides loaded onto MHC class II, which is only expressed on professional antigen presenting cells such as DCs. Several types of CD4 T cells exist. CD4 Th1 cells promote cell-mediated immunity by increasing phagocytosis through secreting cytokines that activate macrophages, promote inflammation, and increase production of antibodies to coat the pathogen's surface. CD4 Th2 cells promote humoral immunity by secreting cytokines that promote B cell differentiation and production of neutralizing antibodies.[36]

CD8 cells

CD8 T cells, also known as cytotoxic T cells, recognize foreign peptides loaded onto MCH class I, which is expressed on most nucleated cells. CD8 T cells are the primary defense against intra-cellular pathogens, particularly viruses. Mature CD8 T cells release toxic granules that include granzyme, perforin, and other molecules that promote apoptosis of the target cell. Both NK cells and CD8 T cells secrete type II IFN, or IFN-γ, which activates macrophages and CD8 T cells. IFN-γ helps induce apoptosis of infected cells, inhibits viral replication, and increases MHC class I presentation of antigens. Activation of nearby macrophages by IFN-γ promotes the phagocytosis and destruction of infected cells.[36]

Humoral immunity

Antibodies, secreted by B cells, mediate humoral immunity via multiple mechanisms of action. First, antibodies can neutralize a pathogen by binding to proteins that mediate entry of the pathogen into a cell. Second, antibodies can coat a pathogen to promote phagocytosis by macrophages. Third, the complement cascade can be initiated by antibody binding. Antibodies are organized into several subclasses: IgA, IgD, IgE, IgG, and IgM. All antibodies are "Y"

shaped and contain at least two heavy chains and two light chains. The variable region (tips of the Y) is formed by VDJ recombination and interacts with a specific epitope. The Fc portion (stem part of the Y) remains constant and interacts with the host immune system. The Fc heavy chain portion of an antibody determines its isotype (IgA, IgD, IgE, IgG, or IgM). Class switching allows various isotypes to be expressed following VDJ recombination in B cells. The variable region of antibodies does not change following antibody class switching. Antibody producing B cells are referred to as plasma B cells.

IgM is the first antibody class to be expressed. IgM exists in the blood as a pentamer joined by disulfide bonds and J chain. This antibody class can bind up to 10 antigen molecules. IgM plays a key role in complement activation by activating the classical complement pathway. IgD is expressed concurrently and following IgM. IgD and IgM are the only antibodies that can be expressed concurrently. IgD exists primarily as membrane bound antibody that functions with IgM to promote B cell activation and maturation.

IgG is the predominant antibody found in the blood. IgG production requires CD4 T cell help. Functions of IgG include activation of complement, chemotaxis, and promotion of phagocytosis. IgG is the only isotype that can cross the placenta, which occurs via the FcRB receptor located on endothelial cells. This isotype has four sub-classes IgG1-IgG4, with different structures and functions. All four classes are capable of neutralizing pathogens. IgG1 and IgG3 also coat pathogens to promote uptake by phagocytosis, promote NK cell killing, and activate complement.[36]

IgA can occur as monomers, dimers, or trimers combined with a J chain and is present in both the serum (as a monomer) and as secretory IgA (sIgA) (a dimer) in mucosal secretions. IgA

occurs in two isoforms. IgA1 is found mostly in the serum, upper respiratory tract and upper digestive tract. IgA2 is found mostly in the lower digestive tract and female genital tract. IgA1 is more susceptible to bacterial proteases than IgA2. The majority of IgA is produced by plasma cells in mucosal epithelium. IgA production requires mucosal stimulation and CD4 T cell help. In contrast to IgG, IgA does not induce inflammation.[39]

IgE binds to mast cells, basophils, and activated eosinophils where it recognizes allergens and parasites. CD4 Th2 cytokines promote IgE development. Activation of mast cells, basophils, and eosinophils by IgE binding to its ligand causes the release of pro-inflammatory mediators, which very quickly induces inflammation. IgE evolved to eliminate parasites from the airway and gut. Allergies occur when an individual makes IgE specific to non-harmful substances, such as pollen.[36]

Mucosal immunity

The mucosa is protected by its own unique combination of innate and adaptive immune responses. Mucosal immunity depends mostly on pathogen-specific sIgA, which is produced locally, and IgG passively transferred from the blood. Immunity mediated by sIgA occurs by neutralizing interactions of the pathogen with the mucosal surface and by intracellular neutralization of pathogens in vesicles.[40] Plasma IgG passively transferred to the mucosal surface by paracellular leakage (transudation) also plays a role in mucosal immunity. Inducing mucosal immunity through vaccination is possible but more difficult than inducing systemic immunity.[41]

The mucosal immune system is comprised of mucosal-associated lymphoid tissue (MALT), which includes gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid

tissue (BALT), nasopharynx-associated lymphoid tissue (NALT), salivary glands, mammary glands, and urogenital glands. The common mucosal immune system (CMIS) provides communication between mucosal tissues and the MALT. CMIS responses can be compartmentalized, with the mucosal site of immune induction stimulating immune responses at some MALT sites but not others. For example, nasal immunization induces antibody responses in the nasal mucosa, salivary glands, and BALT without inducing gut immunity.[41]

Two types of mucosal surfaces exist (Table 3). Type I mucosa is covered by simple columnar epithelium. This type of mucosa is found in the gut, lungs, and cervix. Type I mucosa contains sIgA transport receptors, MALT, and a submucosa with macrophages, lymphocytes, and DCs. Type II mucosa is covered by stratified squamous epithelial cells. This type of mucosa is found in the eyes, mouth, and vagina. Type II mucosa does not express IgA transport receptors, lacks MALT, and has minimal immune cells in its submucosa.[42]

Table 3: Comparison of type I and type II mucosa.[42]

	Type I Mucosa	Type II Mucosa
Location	Gut, lungs, cervix, uterus	Eyes, mouth, vagina, foreskin
Epithelia type	Simple columnar	Stratified squamous
MALT	YES	NO
Submucosa immune cells	Prevalent	Minimal
sIgA receptor	YES	NO
Major antibody	IgA	IgG

sIgA

sIgA is produced by resident plasma cells in mucosal epithelium. sIgA antibodies are predominately dimers joined by one or more J chains and a secretory component (Fig 5). sIgA can also be secreted as trimers with J chains which is called polymeric IgA (pIgA).[43,44] J chain is a 15 kDa polypeptide that covalently binds to the carboxy terminus of IgA and IgM. Antibodies are bound by the polymeric Ig receptor (pIgR) on the basolateral surface of mucosal epithelial cells. This receptor also releases IgM. IgA or IgM bound to the pIgR are transcytosed from the basolateral surface to the apical surface of the cell. The C-terminus of pIgR is degraded intra-cellularly.[40,44] The 80 kDa extracellular portion of pIgR, the secretory component (SC), remains attached to the sIgA or IgM molecule, which provides extra stabilization to the antibody. sIgA occurs as two sub-types sIgA1 and sIgA2. Peripheral plasma cells mostly produce sIgA2 which is more resistant to bacterial proteases than sIgA1.[43]

sIgA secreted into the area surrounding a mucosal surface can block attachment and entry of pathogens and toxins. The Fc portion of sIgA does not induce inflammation of the mucosal epithelium.[45] In addition to blocking pathogen invasion on the exterior of the mucosal epithelium, sIgA can neutralize infection inside endosomes of mucosal epithelial cells. Endosomes transporting sIgA to the apical surface of the epithelium fuse with endosomes carrying a pathogen, such as a virus, and neutralize the pathogen inside the vesicle.[40]

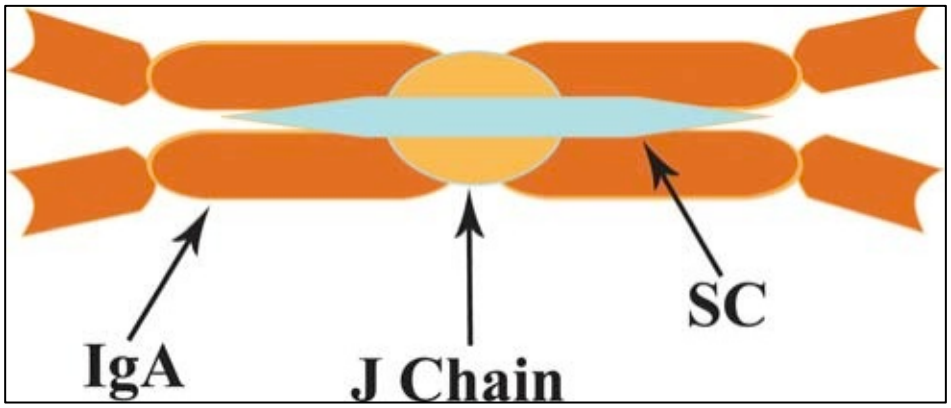


Figure 5: Structure of sIgA. sIgA is a dimer of IgA that contains a J chain for stabilization and a SC component, which is the surface component of the pIgR.[41]

Oral cavity

The oral cavity consists of both the mouth, which is lined with stratified squamous epithelial cells, and several different MALT tissues, including the buccal mucosa, salivary glands, and palatine tonsils. Although the oral mucosa produces cytokines and contains immune cells, the saliva provides the majority of immune defense in this area. Oral mucosa and salivary glands have an extremely high blood flow rate, about 20 times that of muscle, with a large capillary supply. Whole saliva includes secretions from the parotid, submandibular, sublingual, and several other minor salivary glands. Water and electrolyte concentrations of saliva are similar to those of plasma but saliva contains only one tenth of the protein concentration of plasma. Organic compounds found in saliva include antibodies, digestive enzymes, and anti-microbial enzymes. Saliva has a high potassium concentration and is hypotonic.[46]

Salivary glands produce two different types of secretions that combine to form whole saliva. Serous secretions, the main secretion of parotid salivary glands, contain mostly α-amylase. Mucus secretions, the main secretion of minor salivary glands, contain lubricating mucins. Submandibular glands produce both types of secretions. Whole saliva also includes ginigival crevicular fluid that contains antibodies via paracellular leakage from the blood. IgG passively transferred from the blood is typically found in the saliva at levels 800-1,000 fold below that seen in serum.[47,48] Plasma cells producing sIgA are far more prevalent than IgG producing plasma cells in the salivary glands.[46] IgA producing plasma cells are located in the acini of salivary glands. The submandibular gland contains about two-fold more IgA producing plasma cells than the parotid salivary gland. About 40% of salivary gland IgA-producing plasma cells produce IgA2. Plasma cells in the salivary gland tend to be primed in the NALT with minimal contribution of GALT induced B cells.[44] sIgA and IgG concentrations are hard to

measure and vary depending on sample collection and stressors. Unstimulated saliva has roughly three times more sIgA than stimulated saliva. Stressors, such as academic stress, can increase (chronic academic stress) or decrease (acute academic stress) salivary sIgA. Additional factors such as time since last meal, cigarette smoking, and pregnancy can alter sIgA levels in saliva.[44]

Gut

Gut tissue is protected from pathogens by GALT, which consists of lymphoid cells in intestinal villi and Peyer's patches. Each layer of gut epithelia contains a different level of defense against invading pathogens. The outermost epithelial layer contains mostly CD8 T cells to monitor the intestinal mucous epithelium. The lamina propria, located under the epithelial layer, contains B cells, CD4 T cells and macrophages in clusters. The submucosal layer, located under the lamina propria, contains Peyer's patches with 30-40 follicles each. These follicles contain dendritic cells and can develop into germinal centers to allow B and T cell development. Finally, microfold epithelial cells (M cells) exist in the follicle-associated epithelium on top of the Peyer's patches. M cells transport a sampling of antigen from the intestine into the Peyer's patch.[41] Intestinal immunity is mostly driven by M cell antigen sampling.[43]

Genital tract

The genital tract provides a unique mucosal environment that is protected by a combination of type I and type II mucosal epithelia (Table 3). Male and female genital tracts differ in the amount of each mucosal epithelium type. In females, type I mucosa lines the uterus and endocervix, while type II mucosa lines the vagina and ectocervix. In males type II mucosa lines the foreskin with no major area of type I mucosa. Circumcision removes the male foreskin and is thought to prevent disease by removing the genital mucosal epithelium. Due to the lack of

sIgA production at type II mucosal surfaces, neutralizing IgG antibodies are the ideal immune effector mechanism because they do not require local antibody production.[42] Secretions from the female genital tract contain high concentrations of both IgG and IgA. CD8 T cells also provide protection at this site by clearing infected cells.[49]

CMV immune response

The immune response to CMV is diverse and complicated. The major viral antibody targets are envelope glycoproteins. gB (UL55), also known as gCI, is by far the immune-dominant protein in the CMV virion accounting for at least 50% of fibroblast entry neutralizing antibodies. Both systemic and mucosal antibodies are generated against gB in children and adults following CMV infection.[50,51] gB is proteolytically cleaved into a surface component that contains the 110 kDa N-terminus and a 55 kDa C-terminus that contains the transmembrane and cytoplasmic domains. Disulfide bonds occur between the cytoplasmic domains. This protein is very heavily glycosylated with mostly N-linked sugars. Neutralizing antibodies are also generated to the gCII complex of gM and gN which are the most abundant glycoproteins in the CMV envelope. The gCIII complex contains gH/gL/gO and mediates CMV entry into host fibroblast cells.[4,5] An alternative GCIII complex, gH/gL/UL128-UL131, mediates entry into host endothelial and epithelial cells.[26,29] Neutralizing antibodies to this complex were recently identified[52] and components of the complex are emerging as important vaccine candidates. Other CMV proteins that induce strong antibody responses include pp65, IE1, pp150, pp28, and pp71. Passive transfer of CMV antibodies reduces disease in fetal infection and in transplant recipients.[15,53,54]

T-cell immune responses to CMV are variable but are extremely important for viral clearance. In healthy seropositive adults CMV-specific T cells make up a disproportionally large

percentage (~10%) of the CD8 T cell pool. Immune-compromised individuals develop CMV disease once T cells are depleted to predictable levels. In general the immune-dominant CD8 T cell target proteins are pp65, IE1, and IE2, but CD8 T cells specific for the following CMV proteins are documented: gB, gH, pp28, pp50, pp150, US2, US3, US6, US11, UL16, and UL18.[5] CD8 T cell responses can vary significantly between individuals. For example, individuals can develop a CD8 T cell repertoire that recognizes IE1 but not pp65 and vice versa.[4] Overall the CD8 T cell repertoire includes epitopes in 70% of predicted CMV ORFs covering all stages of CMV replication and gene expression.[5]

The CD8 T cell response to CMV expands with age, a phenomenon known as memory inflation. In advanced age CMV-specific memory CD8 T cells can reach over 40% of the total CD8 T cell pool. The reduction in naïve CD8 T cells that occurs with age is thought to contribute to immune senescence and the immune risk phenotype (IRP).[5] IRP is a very serious condition where the CD4:CD8 ratio inverts, which leads to poor immune system function.[1] High CMV-specific CD8 T cell pools may also contribute to reduced success rates for influenza vaccination in the elderly.[5]

Similar to CD8 T cells, CD4 T cells directed against CMV recognize a wide variety of epitopes. gB is the most prevalent CD4 T cell epitope, accounting for about 30% of CMV-specific CD4 T cells. The majority of the CD4 T cell response is to conserved regions of gB and gH. CMV specific CD4 T cells also make up a large proportion, about 9.1%, of the total CD4 T cell population. A decline in CD4 T cells predicts CMV disease in immune compromised individuals.[5]

Although the humoral and cell-mediated responses to CMV are well documented, both responses require an intact innate immune response to develop properly. Most of the research into the innate response to CMV has been done in the mouse model with murine cytomegalovirus (MCMV). In the mouse model TLR9 and TLR3 are vital in host defense against MCMV.[5] TLR3 binds dsRNA, which stimulates the production of type I INF by DCs. TLR9 recognizes dsDNA with unmethylated CpG motifs, which stimulates the production of pro-inflammatory cytokines and NK cell activation.[55] Additionally, gB and gH from human CMV activate the cell surface TLR2 receptor to up-regulate pro-inflammatory cytokine production (Table 4).[5,56] Recognition of gB and gH by TLR2 involves heterodimerization with TLR1 and the presence of CD14 as a co-receptor. Activation of TLR2 by gB and gH induces pro-inflammatory cytokines but not type I IFN.[55]

The major innate immune cells that provide defense against CMV are NK cells. The mouse locus Cmv1 provides a strong example for the importance of NK cells in controlling CMV infection. This gene locus includes the mouse NK cell receptor LY-49H, which recognizes the MCVM protein m157 to activate NK cells to clear viral infected cells. Mice lacking LY-49H are highly sensitive to MCMV infection. Humans with NK cell deficiency develop severe CMV infections supporting findings from the mouse model.[5]

Table 4: TLR activation in CMV infection[1,5,55,56]

TLR	Recognizes	Function
TLR2	gB and gH (cell surface)	Up-regulates proinflammatory cytokines
TLR3	Viral dsRNA (endosomal)	Induces type I IFN Promotes DC maturation Up-regulates proinflammatory cytokines
TLR7	Viral ssRNA (endosomal)	Induces type I IFN Activates NK cells
TLR9	Viral dsDNA (endosomal)	Induces type I IFN Activates NK cells

Viral immune evasion

CMV encodes many proteins that act to evade the host immune system (Table 5). The majority of these proteins either inhibit MHC class I presentation of CMV antigens or inhibit NK cell activation. A number of CMV genes function to inhibit MHC class I. US2 and US11 export MHC class I heavy chain from the ER to the cytosol, where it is targeted to the proteasome and degraded. US3 prevents MHC surface expression by retaining MHC in the ER. US6 blocks TAP, the molecule that transports peptides into the ER lumen and loads them onto MHC I molecules. Blocking TAP retains MHC class I in the ER because MHC class I molecules without peptides are retained in the ER.[4]

CMV evades NK cell activation several ways. First, UL18 encodes an MHC class I homolog to counteract infected cell down regulation of MHC I, which triggers NK cell activation and target cell lysis. Second, UL40 contains a leader peptide that binds to and upregulates surface expression of HLA-E, which also prevents NK cell recognition of the infected cell. Third, UL16 blocks binding of NK cell receptors to activating ligands that are upregulated during CMV infection. Several other CMV proteins prevent NK cell activation including pp65 (UL83), IE2 (UL122), UL141, and UL142.[5] Clinical isolates prevent NK cell activation better than lab strains, suggesting that viral NK evasion genes were lost during attenuation of common lab strains.[4]

Table 5: CMV-encoded immune evasion genes[1,5,57]

Gene	Class	Function
US2	Inhibit MHC Class I or II	Export MHC class I from ER to cytosol Target MHC class II for degradation
US11		Export MHC class I from ER to cytosol
US3		Retain MHC in ER
US6		Inhibits TAP
UL111A	Homologs of cytokines and cytokine receptors	IL10 homolog: down regulates MHC class I and II, impairs acidification
UL144		TNF receptor homolog
UL146		IL8 homolog
UL33	Homolog of G protein coupled receptors that block the inflammatory response	
UL78		
US27		
US28		
UL16	NK cell evasion	Prevents NK cell activation
UL83		Prevents NK cell activation
UL141		Prevents NK cell activation
UL142		Prevents NK cell activation
UL18		MHC class I homolog Binds to inhibitory NK cell receptor LIR-1
UL40		Leader peptide binds to HLA-E to increase cell surface expression
UL122		Blocks type I IFN induction
UL36	Inhibit apoptosis	
UL37		
TRS1	Inhibit PKR	
IRS1		

Additional CMV genes encode immune receptor analogs. UL33, UL78, US27, and US28 encode G-protein coupled seven transmembrane receptors that bind to various chemokines. UL144 encodes a TNF receptor. CMV encodes several chemokine and cytokine homologs such as UL111a (IL-10 homolog) and UL146 (IL-8 homolog). Apoptosis is blocked by UL36 and UL37.[5] CMV synthesizes dsRNA during infection, which stimulates the host cell PKR response. CMV evades the PKR response by producing dsRNA binding proteins, TRS1 and IRS1, which reduce PKR activation. Deletion of TRS1 and IRS1 from CMV leads to attenuation due to impairment of replication by PKR activation.[38] Complement-mediated lysis is evaded by the incorporation of human complement-inhibitory proteins, such as CD55, in the CMV envelope.[58]

Natural history

Transmission of CMV occurs across the placenta, from breast milk, blood transfusions, and contact with infected fluids at the oral and genital mucosa.[3] CMV disease varies based on the immune competence of the host. Clinical signs, symptoms, and laboratory evidence of CMV in blood and tissues guide diagnosis. CMV viremia cannot solely be used for diagnosis because asymptomatic individuals can have detectable viremia.[5] Asymptomatic individuals shed virus in their urine, saliva, breast milk, and genital secretions for varying time intervals. Virus shedding in the urine of adolescents lasts for less than six months,[4] with brief intermittent shedding in the saliva and genital secretions. Women of childbearing age are most likely to shed CMV in their genital tract.[59] In contrast, young children shed CMV in their urine and saliva for an average of 18 months.[3] Following primary infection CMV becomes latent in a poorly understood immune cell reservoir that includes monocytes, macrophages, and dendritic cells. Latently infected cells carry 2-13 genome copies per cell and occur in 0.004-0.01% of circulating mononuclear immune cells. CMV most likely frequently reactivates from latency but does not cause disease unless an

individual becomes immune-compromised. Transplant and HIV/AIDS patients develop CMV disease when T cell immunity becomes impaired.[5]

Vaccine development

The Institute of Medicine declared the development of a CMV vaccine as a national priority in 2000.[60] CMV vaccine development has been an active field of research for over 40 years. A variety of approaches to induce CMV immunity have been tested in clinical trials and animal models. To date the most successful vaccine tested in clinical trials is the gB/MF59 subunit vaccine (see below) that provided protection against CMV infection to 50% of women of childbearing age.[61] Although these results are promising in the goal of a prophylactic CMV vaccine, better vaccine efficacy is needed for a successful vaccine.

Neutralizing activity using fibroblasts has served as a presumed correlate of protection. gB became a focus of vaccine development because it is the major neutralizing target for fibroblast entry. Recently, it was reported that sera from naturally infected individuals are considerably more potent in neutralizing CMV infection of epithelial cells versus fibroblast cells.[62,63] Seropositive adults have an average fibroblast entry neutralizing titer of approximately 1:64, but an average epithelial entry neutralizing titer of approximately 1:1,000. The mean ratio of epithelial to fibroblast entry neutralizing titers is 48-fold. Towne and gB/MF59 vaccine recipient sera have fibroblast entry neutralizing activity similar to natural infection but much lower epithelial entry neutralizing activity than natural infection.[62]

Potential need for multiple vaccines

Due to the nature of CMV disease multiple vaccines may be necessary to protect vulnerable populations. Neutralizing antibodies that cross the placenta via IgG receptors provide

the majority of the immune defense and control of congenital disease.[11,16,64] Clinical trial data suggests that administration of CMV hyperimmunoglobin during pregnancy decreases congenital disease.[15] These data suggest that a successful CMV vaccine to prevent congenital infection needs to induce high neutralizing antibody titers in women of childbearing age. Ideally a vaccine should induce antibodies capable of blocking CMV entry into the oral mucosa. If protection at the maternal oral mucosa is not possible, neutralizing antibodies may prevent dissemination to the placenta to decrease transmission of CMV to fetus. At a minimum, neutralizing antibodies crossing the placenta can reduce fetal disease.

In most cases of CMV disease in immune-compromised adults, the individual is naturally seropositive or, if seronegative, likely to become infected via blood products or donor tissues. Hence, neutralizing antibodies in the oral mucosa would not be relevant. Therefore, CMV disease in immune-compromised adults, in contrast to congenital disease, closely correlates with a reduction in functional cell mediated immunity. Prior to the development of anti-retroviral therapy CMV disease was extremely prevalent in HIV patients with less than 100 CD4 T cells per μl of blood. CD4 T cell counts can also be used to predict CMV disease in SOT recipients. Adoptive transfer of CMV-specific T cells decreases viral replication in HSCT recipients. These observations suggest that T cell immunity is more important in the control of CMV disease in immune-compromised populations than neutralizing antibodies.[5] However, a strong gB-specific antibody response can also decrease viremia and duration of anti-viral therapy, as demonstrated by a recent gB/MF59 phase 2 clinical trial in SOT patients. gB antibody titers prior to transplantation inversely correlated with duration of viremia post transplantation.[65] gB specific T cell responses were not determined in study participants but gB is not a strong T cell

immunogen so reduced disease in vaccinated subjects is presumably due to gB-specific antibodies.

Multiple vaccines specific to each CMV disease process may be necessary instead of one universal CMV vaccine to cover all vulnerable populations. One vaccine that targets high titer neutralizing antibodies to prevent congenital disease and a second vaccine that targets T cells for immune-compromised patients may be necessary. Alternatively, a single vaccine could stimulate both arms of the adaptive immune system to protective levels, but this may not be possible. Recent data on the gB/MF59 vaccine suggests that strong vaccine-specific antibody responses may be protective in both prevention of congenital and transplant CMV disease. Although promising, the gB/MF59 vaccine is not ideal. Addition of epithelial entry specific targets may drastically increase the immunogenicity of a subunit vaccine approach.

Targeting mucosal immunity

Since CMV initially infects a host at mucosal membranes, an ideal vaccine to prevent maternal infection will induce a strong mucosal immune response.[3] Natural infection induces measurable gB-specific IgG antibodies in the saliva of about half the seropositive adults tested.[51] Unfortunately traditional intra-muscular vaccine administration does not typically induce the mucosal immune system. Immunization delivered at mucosal sites, such as intra-nasal immunization, provides the best mucosal immune protection. Intra-nasal immunization induces sIgA in the salivary glands, respiratory tract, genital tract, and intestine. Compared to immunization at other mucosal sites, such as the rectum and female genital tract, intra-nasal immunization induces higher systemic antibody levels.[49]

Ideal vaccines induce both systemic and mucosal immunity. Prime/boost vaccine strategies offer an attractive mechanism to achieve this high level of immunity. In general priming with a mucosal administered vaccine followed by a parenteral vaccine boost should provide the best vaccine response because the mucosal prime will activate both mucosal and systemic lymphocytes but a parenteral prime may not sufficiently prime the mucosal immune system.[49]

Animal models

Animal models are of limited use in CMV vaccine development because CMV is extremely species specific and does not cause the same disease process across species; nevertheless animal studies are necessary for pre-clinical vaccine development. Additionally, a considerable amount of CMV immunology was deduced based on animal models, particularly MCMV. Unfortunately, MCMV does not cause congenital infection but vaccine studies in the mouse model shed light on immunogenicity of various viral components. The mouse immune system and its reaction to MCMV are fairly well characterized. Guinea pig CMV (GPCMV) can cause congenital infection so it provides an animal model to study fetal protection for CMV vaccine development. When directly inoculated rhesus CMV (RhCMV) infects the fetus with similar sequelae to human CMV but transmission during pregnancy has not been demonstrated. Disadvantages of this model include increased cost of the animals, limited availability of seronegative animals, and a limited understanding of the rhesus immune system.

Mouse model

Many different MCMV vaccines have been tested in mice. Initial MCMV vaccine research involved formalin inactivated MCMV virions. This vaccine was protective against lethal challenge in the majority of mice tested but did not prevent viral replication in the salivary

gland. Protection against lethal challenge correlated with the neutralizing antibody response in individual mice. Passive transfer of MCMV hyperimmune serum also protects against lethal challenge, supporting the observations of the previous study. Interestingly, the hyperimmune serum did not reduce viral replication in visceral organs. Adoptive transfer to CD8 T cells also protects against MCMV disease. Live attenuated vaccines have also been studied in the mouse model. A temperature sensitive MCMV strain induces protective B and T cells responses against lethal challenge. This vaccine was effective in B cell knock out mice indicating that productive CD8 T cell responses were induced by live attenuated vaccine immunization.[4]

Viral vectored subunit vaccines to the MCMV homolog of gB but not gH are protective against lethal challenge. Comparison of full-length gB to truncated secreted gB DNA vaccine found that the secreted form of gB was more immunogenic. These DNA vaccines induced different types of antibodies. Full length gB DNA vaccination induced mostly IgG2a antibodies while secreted gB DNA vaccination induced mostly IgG1 antibodies. Vaccination with a pp65 homologue (pp83) DNA vaccine induced B and T cell responses. The gB and pp65 DNA vaccines do not interfere with each other when administered at the same time.[4]

DNA immunization with pp83 and M84, a non-structural MCMV protein, significantly reduced viral replication in the spleen but did not reduce viral replication in the salivary gland. Viral replication in the spleen was controlled by induction of CD8 T cells. A DNA vaccine including plasmids for pp83, M84, gp34 (a strong CD8 T cell epitope), and a pool of 10 plasmids for non-structural, capsid, and matrix proteins, that are not individually protective, followed by a boost of formalin inactivated virions formulated in alum, reduced viral replication in both the spleen and salivary glands. Inclusion of minor antigen plasmids increased the immunogenicity of the vaccine by broadening the CD8 T cell vaccine repertoire to cover multiple sites and stages

of viral replication. DNA immunization alone induced high levels of CD8 T cells but the inactivated virion boost was necessary to protect vaccinated mice against viral replication in the spleen and salivary gland. The neutralizing antibodies induced by the inactivated virion boost work synergistically with the DNA immunization-induced CD8 T cells to control viral replication throughout the host.[66]

A follow-up study demonstrated that DNA immunization with plasmids for IE1, M84, and gB with a formalin-inactivated virion boost also reduces viral replication in the spleen and salivary gland. Additionally, immunized mice had detectable IgA in their lungs a week earlier than control mice following intra-nasal challenge. Viral titers in the lungs of immunized mice challenged intra-nasally were several fold lower than controls and immunized mice had reduced viral replication in other organs, such as the liver and spleen. These data suggest that a systemic DNA prime/inactivated virion boost vaccine induces mucosal immunity.[67]

In order to target mucosal immunity a recombinant vesicular stomatitis virus (rVSV) expressing MCMV gB was designed (rVSV-gB). The vaccine was well tolerated by vaccinated animals. After a single intra-nasal immunization with rVSV-gB all immunized mice developed serum antibodies and a CD8 T cell response to gB. Following intra-peritoneal challenge viral titers in the lungs of vaccinated mice were reduced compared to control mock vaccinated mice. These data support future development of this vaccine candidate.[38]

A second viral vectored vaccine designed to induce mucosal immunity is the replication deficient adenovirus vaccine platform. Expression of MCMV gB or gH in this vector induces systemic and mucosal immunity to mice immunized intra-nasally. In order to target multiple T cells epitopes, Ad-gBCMVpoly was designed to 46 human CMV T cell epitopes. This vaccine

induces strong B and T cell responses in immunized mice and reduced infection with a challenge recombinant vaccinia virus expressing human CMV gB and IE1.[38]

Recombinant modified vaccinia Ankra (rMVA) is a viral vaccine vector capable of safely delivering multiple target genes. These vectors are able to overcome pre-existing immunity from prior small pox vaccination with vaccinia virus. rMVA expressing CMV gB (gB-MVA) induces gB antibodies in mice at equivalent levels of natural CMV infection in humans. A trivalent rMVA expressing gB, pp65, and IE1 also induces high B and T cell immunity to gB in mice with a positive safety profile.[38]

Guinea pig model

GPCMV causes fetal infection with a similar disease process as human CMV, including inner ear pathology. Immunization with tissue culture attenuated GPCMV protects against lethal challenge and reduces fetal infection.[4] Vaccination with GPCMV BAC DNA also reduced pup mortality and decreased maternal viral load.[68] A glycoprotein subunit vaccine that contains the GPCMV gB, gM, and gN protects against pup mortality. Several other studies investigated the protective capacity of secreted gB. DNA vaccines for gB and the pp65 homolog GP83 reduced fetal disease in a gB-dependent manner. Baculovirus expressed gB with Freund's adjuvant also reduced congenital infection. These studies support the development of subunit vaccines that include secreted gB.[4] GP83 incorporated into a alpha virus vaccine (VRP-GP83) reduced viral load in pregnant guinea pigs during a third-trimester challenge.[69]

Rhesus model

An rMVA vaccine expressing RhCMV gB, pp65, and IE1 (rMVA-rh gB/pp65/IE1) was tested in this model with and without DNA priming for the same three RhCMV proteins. A

single DNA immunization primed the immune response for rMVA-rhgB/pp65/IE1.[38] DNA prime (intra-muscular and intra-dermal) followed by a single rMVA boost (intra-muscular) induced higher antibody titers and T cell responses then either DNA or rMVA alone. A second rMVA boost further increased RhCMV specific antibody titers but not T cell responses. Following the second rMVA boost, animals were challenged with a lab strain of RhCMV. Compared to controls, prime/boost vaccinated animals had a reduced viral load.[70]

Formalin-inactivated RhCMV virions are immunogenic in monkeys. Therefore, formalin-inactivated RhCMV virions (subcutaneous) were used as a boost following a DNA prime of gB, pp65, and IE1 (intra-muscular and intra-dermal). Inactivated virions successfully boosted immune responses to the DNA vaccine. Robust B and T cell responses were induced in vaccinated monkeys. Animals were then challenged subcutaneously with a lab strain of RhCMV that does not cause shedding in the saliva of naïve, unvaccinated animals. Vaccinated animals demonstrated reduced viral replication at the site of viral challenge.[71]

DNA priming with RhCMV gB, pp65, and IE1 (intra-muscular and intra-dermal) followed by formalin-inactivated virion (intra-muscular) or rMVA-rhgB/pp65/IE1 (intra-muscular) boost was tested in this model with epithelial tropic virus challenge. Both prime/boost strategies induced neutralizing antibodies in vaccinated monkeys prior to viral challenge. Following challenge with epithelial cell tropic RhCMV, all vaccinated monkeys shed virus in their saliva, even though vaccinated monkeys had reduced plasma viral loads. Therefore, salivary shedding was not impacted by the presence of systemic neutralizing antibodies. Several monkeys with strong pp65 T cell responses had lower viral titers in their saliva suggesting the T cell responses control viral shedding in the saliva.[72]

Live-attenuated vaccines

Early CMV vaccine development focused on the use of live-attenuated vaccines. Serial passage through fibroblast cells is the most common method to attenuate virus. During adaptation to growth in fibroblast culture the virus will randomly loose genes not necessary for propagation in fibroblast cells. Therefore, during attenuation CMV spontaneously deletes sections of its genome.

AD169

AD169 was the first live-attenuated CMV vaccine tested in clinical trials. Strain AD169 was originally isolated from human adenoidal tissue. The virus was then attenuated by 54 passages in four different human fibroblast cell lines.[4] The UL131 gene in AD169 contains a single nucleotide insertion that prevents gene expression. Due to this mutation AD169 is not epithelial or endothelial cell tropic.[26] Initial safety studies, that included 46 CMV seronegative adults, found that AD169 was safe and attenuated since vaccine virus was not shed by trial participants. All but one trial participant developed CMV-specific antibodies following AD169 vaccination but this immunity waned over time. CMV seropositive individuals did not respond to the AD169 vaccine. Due to poor immunogenicity AD169 was not pursued as a potential CMV vaccine.[4]

Towne

The Towne live attenuated CMV vaccine has been tested safely in almost 1000 clinical trial participants.[73] The CMV Towne strain was isolated from the urine of a two month old child with congenital CMV disease, then passaged 125 times in WI-38 human diploid fibroblast cells. The safety profile of Towne is similar to that of AD169. [74] The UL130 gene in Towne has a frame shift mutation resulting from a two-base pair insertion that causes the protein to be rapidly

degraded.[33] The loss of functional UL130 prevents Towne from entering epithelial and endothelial cells.[26] Towne induces lower epithelial neutralizing antibody titers than natural infection[62] and does not induce detectable gB antibodies in the saliva.[75]

CMV seronegative Towne vaccine recipients develop CMV specific antibodies but the vaccine does not boost pre-existing antibodies in seropositives. Towne virus could not be isolated from any vaccine recipients, even following immune suppression for transplantation. CMV-specific antibodies induced from intra-muscular injection of the Towne vaccine have similar specificities to those induced by natural infection but the antibodies decrease over time compared to natural infection.[4] Antibody avidity maturation following Towne vaccination is similar to that of natural infection.[76] Towne vaccine recipients also develop CD4 and CD8 T cells responses. Unfortunately, intra-nasal administration of Towne in CMV seronegative adults did not induce CMV-specific antibodies.[4]

Multiple clinical trials of the Towne vaccine in kidney transplant patients have been conducted. Compared to healthy adults, kidney transplant recipients developed reduced B and T cell responses following Towne vaccination. Placebo controlled Towne vaccine trials failed to show protection from CMV infection post transplantation but overall CMV disease was decreased. Incidence of severe CMV disease in seronegative kidney transplant recipients that received a CMV positive kidney was decreased to levels seen in seropositive kidney transplant recipients. A combination of three studies found an 86% decrease in severe CMV disease in seronegative recipients of seropositive kidneys but only a 30% decrease in overall CMV disease. These data demonstrate that the Towne vaccine can be at least as protective as natural protection in the SOT population.[4]

The Toledo strain of CMV, originally isolated from the urine of a congenitally infected infant, causes symptomatic disease when 10 or 100 plaque forming units (PFU) are administered subcutaneously. Following administration Toledo can be cultured from blood, urine, and saliva.[77] Neutralizing antibodies are generated to Toledo in seronegative individuals at similar levels when inoculated with 10 or 100 PFU of Toledo. Titers of neutralizing antibodies originally generated to Toledo were three to five-fold higher when sera were tested against Toledo vs. Towne.[78] Toledo challenge of 100 PFU is the limit of natural infection protection since the next dose, 1000 PFU of Toledo induced symptomatic disease in 100% of seropositive individuals challenged.[77]

Towne vaccinated individuals challenged with 10 PFU of Toledo one year post vaccination were protected from CMV infection, with no evidence of disease and negative viral cultures.[77] Following 10 PFU of Toledo challenge, no increase in fibroblast entry neutralizing titers to Towne or Toledo were detected.[78] 60% of Towne vaccinated individuals challenged with 100 PFU of Toledo developed positive CMV viral cultures post challenge. Of the individuals with positive viral cultures 75% had laboratory evidence of CMV disease and the remaining 25% developed severe CMV disease. This is in contrast to control CMV seropositive individuals challenged with 100 PFU of Toledo that showed no evidence of CMV disease.[77] Neutralizing antibodies increase 20-fold to both Towne and Toledo in response to the 100 PFU Toledo challenge in Towne vaccinated individuals that were seronegative prior to Towne vaccination.[78] Based on these data the Towne vaccine provides measurable protection against CMV challenge but at a lower level than natural infection.[4,77]

Another placebo-controlled study was conducted to determine if the Towne vaccine protects women with children in daycare against CMV infection. This population was chosen

because young children shed CMV in their urine and saliva for an average of 18 months which increases the attack rate of CMV in their mothers.[3] For this study women with children in daycare under three years of age and shedding CMV were recruited. Both seropositive and seronegative women were randomly and blindly assigned either placebo or the Towne vaccine (500 PFU intra-muscularly) and followed until their children stopped shedding CMV or until seronegative women demonstrated acquisition of natural infection. An equal number of seronegative women received the placebo or Towne vaccine. All seronegative Towne recipients seroconverted following vaccination. 42% of Towne recipients developed subsequent CMV infection, but only 38% of infected women shed virus. Of these women 63% shed the same strain of virus as their child. These results were very similar to the placebo group. 47% of the women in the placebo group acquired natural infection, and 44% of these women shed the same viral strain as their child. In contrast, only 7% seropositive women shed the same virus as their child during the course of this study. Overall this study demonstrated that natural infection provides better protection against CMV acquisition by women with young children in daycare compared to the Towne vaccine.[79] At this dose Towne showed no evidence of protection. However, the dose was fairly low (500 PFU) compared to other studies (up to 6000 PFU), so at a higher dose the Towne vaccine may be more protective in this population.

Towne/Toledo Chimeras

The Towne vaccine provides some protection from CMV disease in transplant patients but is not as effective as natural infection in prevention of infection. During the attenuation process, Towne acquired multiple mutations and deletions but it is not known which of these are involved in Towne's attenuated phenotype. Towne/Toledo chimeras were created as vaccines that are attenuated compared to wild type Toledo but not as attenuated as Towne. These

vaccines were developed based on the assumption that Towne, although safe, is too attenuated to be immunogenic and that Toledo is not attenuated enough to be a safe vaccine.[80] Four chimeric viruses were developed from previously described overlapping cosmid clones[75,81] from Towne and Toledo. The combination of all four chimeric viruses incorporates the entire Towne genome, except the UL/b' region (Fig 6). The Toledo UL/b' region, which contains 19 genes missing from Towne, was included in all four chimeric viruses because it was assumed that this region contained genes not present in Towne and that the right combination of Towne and Toledo genes will be safe and immunogenic. An additional advantage of the Toledo UL/b' is that his region is present in an inverted orientation in each chimeric virus so it can serve as a genetic marker for the vaccine.[80]

The chimera vaccines were tested in CMV seropositive adults in a double blinded, randomized, placebo controlled trial with five groups: placebo or 1000 PFU of one of the four chimeras subcutaneously. All four chimeras were well tolerated by study participants and attenuated compared to Toledo. Chimeric viruses were not isolated by culture or PCR. None of the four chimeric viruses stimulated antibody or T cell responses, suggesting that the chimeric viruses cannot boost pre-existing CMV immunity. This study demonstrated that the chimeric viruses are safe in seropositive adults but future studies are necessary to determine safety and efficacy in seronegative adults.[80] A new clinical trial is planned to test all four chimeras in CMV seronegative adult males.

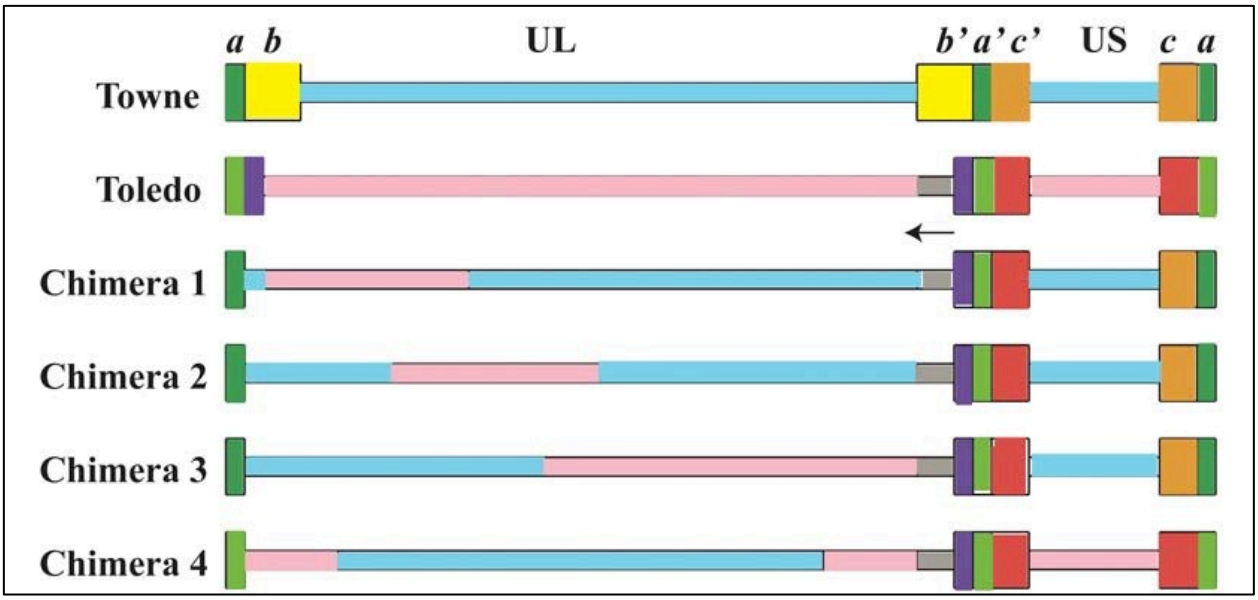


Figure 6: Schematic of Towne/Toledo chimeric viruses. Four chimeras were created so that overlapping sections of the Towne strain replaced sections of Toledo. The UL/b' region of Toledo (grey) is present in each chimera. Towne UL and US regions are shown in blue, *a* sequences are shown in dark green, *b* sequences are shown in yellow and *c* sequences are shown in orange. Toledo UL and US regions are shown in pink, *a* sequences are shown in light green, *b* sequences are shown in purple, and *c* sequences are shown in red.[80]

Subunit vaccines

The gB/MF59 subunit vaccine is currently the most promising CMV vaccine under development. The gB in this vaccine is the gB sequence from Towne expressed in Chinese hamster ovary (CHO) cells. The vaccine protein is modified so that it is not proteolytically processed and lacks the transmembrane domain so it is secreted from CHO cells. gB is purified from recombinant CHO cell supernatants and mixed with the MF59 adjuvant.[4] MF59 is a novel squalene-in water emulsion that targets CD4 Th2 T cells to promote antibody responses.[82,83]

The gB/MF59 vaccine has a positive safety profile from multiple clinical trials. Safety was initially evaluated in CMV seronegative adults in a randomized, double-blinded, placebo-controlled study comparing gB/alum, gB/MF59, and placebo administered on a 0, 1, and 6-month schedule. The vaccine was well tolerated by all study participants. gB antibody responses were analyzed two weeks after the third immunization. Antibody responses to gB were higher in vaccinated individuals than CMV seropositive controls. gB/MF59 was found to be more immunogenic than gB/alum. Subsequent clinical trials demonstrated that 5 μg of gB/MF59 induced equivalent antibody responses as 30 μg of gB/MF59 and that a 0, 1, and 6-month vaccination schedule was more effective than 0, 1, 2-month or 0, 1, 4-month schedules. Studies with gB/MF59 have also demonstrated that the vaccine does not induce auto-immunity. Unlike live-attenuated vaccines, the gB/MF59 boosts antibody responses in CMV seropositive adults. Antibodies induced by the gB/MF59 neutralize multiple clinical isolates.[4] Avidity maturation of gB/MF59 antibodies is similar to that of natural infection.[76]

A small trial of gB/MF59 (n=15) was conducted in toddlers age 12-35 months at a 20 μg dose on a 0, 1, 6-month schedule. These children did not attend large group day care so they were at a low risk for acquiring CMV during the follow up period. Initially, six children were

vaccinated to determine safety. Following a positive safety profile, the remaining twelve children were vaccinated in a randomized, double blinded, placebo controlled (hepatitis A vaccine) trial at a 3:1 ratio. The vaccine was well tolerated by all study participants. Natural CMV infection occurred in two gB/MF59 vaccinated children, one after the first vaccination, another after the second vaccination. gB titers increased following each vaccination, similar to vaccinated adults. The vaccine induced six-fold higher antibody titers in toddlers than adults that received the same vaccine,[84] but the clinical importance of higher antibodies in titers in toddlers is unknown.

A phase II randomized, double-blind, placebo-controlled trial was conducted with the gB/MF59 vaccine (20 μg) on a 0, 1, 6-month schedule given to women with young children. CMV seronegative women between the ages of 14-40 years were enrolled within one year of giving birth. 234 women received the gB/MF59 vaccine and 230 women received the placebo. Trial participants were screened for CMV infection every three months for 42 weeks post vaccination. CMV infection was confirmed within one moth via culture and/or PCR.[61]

None of the trial participants developed symptomatic CMV disease. The overall vaccine efficacy determined by this study was 50% with 8% of the vaccinated group and 14% of the placebo group developing evidence of CMV infection. Rate of CMV infection per 100 person years was reduced from 6.6 in the placebo group to 3.3 in the vaccinated group (Table 6). Placebo recipients became infected with CMV at a statistically significant ($p=0.02$) higher rate than vaccine recipients.[61] Interestingly, an earlier study found detectable gB specific IgG antibody in the saliva of 100% of vaccinated individuals and gB specific IgA in the saliva of 50% of vaccinated individuals.[51] Levels of gB antibody in the saliva of women in this study

have not been determined but it is possible that the women protected from CMV infection had measurable gB salivary antibodies.

Table 6: Results of gB/MF59 phase 2 trial[61]

	Incidence of Infection	Rate per 100 person years
Vaccine	18/225 (8%)	3.3
Placebo	31/216 (14%)	6.6

Women who became pregnant during the follow up period were monitored for CMV infection and congenital disease in their newborns. Time to pregnancy and pregnancy outcome were the same between the two groups but a larger percentage of the placebo group became pregnant. A total of four congenital infections were documented, 1 of 81 (1%) of infants in the vaccine group and 3 of 97 (3%) of the placebo group. Of these infants only one in the placebo group was symptomatic at birth. The remaining infants were asymptomatic at birth and did not develop sequelae 3-5 years later (Table 7). All women with congenitally infected infants developed primary CMV infection during pregnancy. The rate of congenital infection in the vaccine group versus the placebo group was not statistically significant ($p = 0.41$) but that may be due to the low sample size.[61] Although the outcome of this trial is promising, a more effective vaccine is needed.

Table 7: Pregnancy outcome of vaccine participants[61]

	Vaccine	Placebo
Pregnancies	80/225 (36%)	97/216 (45%)
Live births	79/97 (81%)	95/118 (81%)
Spontaneous abortion	14/97 (14%)	12/118 (10%)
Premature infant	12/81 (15%)	15/97 (15%)
Congenital CMV	1/81 (1%)	3/97 (3%)
Asymptomatic	*1/1 (100%)*	*2/3 (67%)*
Symptomatic	*0/1 (0%)*	*1/3 (33%)*

DNA vaccines

CMV specific DNA vaccines are protective in mice against lethal MCMV challenge (see above: Mouse models). Two DNA vaccines, a bivalent vaccine of gB and pp65 and a trivalent vaccine of gB, pp65, and IE1, have been studied in clinical trials. The gB expressed by this vaccine is truncated and secreted, similar to the gB used in the gB/MF59 vaccine. As a safety feature the putative kinase of pp65 is mutated in the DNA vaccine plasmid. Mutation of the protein kinase domain of pp65 does not alter its immunogenicity in animal models.[4]

In the bivalent VCL-CB01 vaccine, the gB and pp65 are based on strain AD169 and the plasmids are formulated with a poloxamer CRL1005 adjuvant and benzalkonium chloride. In a phase 1 clinical trial an equal number of CMV seropositive and seronegative adults were immunized intra-muscularly with 1 or 5 mg of DNA on a 0, 2, 8-week schedule or 5 mg on a 0, 3, 7, 28-day schedule. The vaccine was well tolerated by study participants. Vaccination induced gB antibody responses in seronegative recipients but did not boost gB antibody levels in seropositive recipients greater than two fold over pre-vaccination baseline. T cell responses to pp65 were induced in seronegative and seropositive participants. gB T cell responses were induced in seronegative subjects. Overall, VCL-CB01 was immunogenic in 45.5% of seronegatives and 25% of seropositives at 16 weeks post immunization. At 32 weeks post vaccination 68% of seronegative recipients had measurable memory T cells. The vaccine induced T cell responses more effectively than antibody responses (Table 8).[85]

Table 8: Immunogencity of VCL-CB01 at week 16[85]

	Sero Negative	Sero Positive
gB antibody response	22.2%	0%
pp65 T cell response	37.5%	22.2%

A phase II randomized, double blinded, placebo controlled study of VCL-CB01 to determine safety and efficacy in HSCT patients was recently completed. This trial involved two groups of HSCT participants, donor/recipient pairs (D/R arm) and recipient (R arm); HSCT recipients in both arms were CMV seropositive prior to transplantation. Donors in the D/R arm were immunized 9, 6, 2-days pre-transplantation. Recipients in both arms were immunized 3, 5-days pre-transplantation and 3, 6, 12, 28-weeks post-transplantation. Preliminary data from the trial demonstrated an increase in gB and pp65 T cells but not gB antibody at days 56 and 84 post-transplant in the R arm compared to placebo. Vaccine recipients in the R arm of the trial demonstrated reduced occurrence and recurrence of CMV as measured by PCR, reduced viral load, and reduced time to initial reactivation. Based on the interim data enrollment of D/R arm was discontinued because of limited enrollment and positive results from the R arm.[86] At this time data from the complete trial are not published.

A phase I trial in CMV seronegative adults has also been completed using VCL-CT02, a trivalent DNA vaccine using AD169 sequence of pp65, IE1, and gB formulated in phosphate buffered saline. Kinase domains of pp65 and IE1 were mutated to remove kinase activity and the gB in the vaccine is the secreted form used in previous DNA vaccine formulations. Unlike

VCL-CB01, the VCL-CT02 vaccine trial involved a DNA prime followed by Towne boost. VCL-CT02 was well tolerated by all volunteers regardless of intra-dermal or intra-muscular vaccination. Trial participants boosted with 3000 PFU of live Towne vaccine subcutaneously reported similar adverse events as participants that received Towne alone, which were all minor. Urine cultures post-Towne boost were all negative for Towne.

The immunogenicity of VCL-CT02 DNA prime was poor with only 1 of 5 (20%) intra-muscularly vaccinated participants and 2 of 11 (18%) intra-dermally vaccinated participants developing pp65, IE1 and/or gB T cell responses. No study participants developed gB antibodies. Vaccine induced immunity was similar when participants were immunized on a weekly (0, 7, 14-days) or monthly (0, 4, 8-weeks) schedule. Prior to Towne administration, 60% of intra-muscularly immunized and 20% of intra-dermally immunized participants had detectable memory T cells suggesting that VCL-CT02 primed memory T cells.

Towne boosts were given as 3000 PFU subcutaneously either 11-13 months post-DNA vaccination (DNA/Towne +12) or 77 days (2.5 months) post-DNA vaccination (DNA/Towne + 2). In the group that received Towne an average of one-year post vaccination (DNA/Towne +12) the time to detectable gB antibody, pp65 and gB but not IE1 T cell activity was reduced compared to participants that received Towne only (14 days vs. 28 days; Table 9). Trial participants that received Towne 77 days post vaccination (DNA/Towne +2) did develop T cell responses earlier than Towne only controls but the median time to immune response was not significant (Table 9). Median peak immune responses in the two DNA vaccine groups and the Towne alone group were not statistically different. Overall the results of this study suggest that the VCL-CT02 vaccination leads to a faster but not necessarily higher immune response to the Towne vaccine.[87]

Table 9: p values of the time to first response of DNA vaccination with Towne boost compared to Towne alone[87]

	gB antibody	pp65 T cells	gB T cells	IE1 T cells
DNA/Towne + 12	0.03*	0.02*	0.03*	0.113
DNA/Towne + 2	0.97	0.13	0.97	0.56

*=Significant difference

Recombinant virus vaccines

Vectored vaccines are a method to deliver subunit vaccines in the context of a non-replicating viral vector with a positive safety profile. Two viral vectors have been studied in CMV vaccine development, canarypox and alphavirus.[4]

ALVAC

The canarypox vector, ALVAC, replicates in avian species but not in mammalian cells, which makes it safe for human use. Poxvirus genomes are very large and allow introduction of DNA for multiple genes. ALVAC-gB is immunogenic and safe in animal models. Initial clinical trials found that ALVAC-gB was weakly immunogenic on its own so subsequent trials used prime boost strategies.[88] In CMV seronegative adults three doses of ALVAC-gB induces low antibody titers. In contrast, CMV seronegative adults that received ALVAC-gB prime on

days 0 and 30 then a Towne boost at day 90 developed antibody titers similar to that seen following natural infection and better than those that received Towne following a prime with ALVAC expressing rabies glycoprotein.[89] A second prime boost trial in seronegative adults failed to show a priming response for ALVAC-gB in the context of a gB/MF59 boost.[90] Additionally, ALVAC-pp65 induced CD8 T cells comparable to natural infection in seronegative adults without a boost.[91]

AlphaVax

Venezuelan equine encephalitis (VEE) is an alpha virus that has been genetically engineered as a CMV vaccine (AlphaVax). This is a bivalent vaccine with one vector that expresses the Towne gB and a second vector that expresses a pp65/IE1 fusion protein. Studies of this vaccine in the mouse model show promising immogenicity.[4,92,93] A recent phase I trial of AVX601, a two component alpha virus vectored gB, pp65/IE1 fusion protein vaccine, in CMV seronegative adults showed that the vaccine induces B and T cell responses following three vaccinations in >97% of trial participants. The vaccine was well tolerated by all study participants. These results support further clinical trials of this vaccine.[38,94]

Vaccines induce minimal epithelial entry neutralizing activity

Sera from naturally positive adults neutralize entry of CMV into epithelial cells at titers an average of 48-fold higher than fibroblast cells. Unfortunately, epithelial entry neutralizing activities of sera from Towne and gB/MF59 vaccine recipients are 28-fold and 15-fold lower, respectively, than natural infection. Fibroblast entry neutralizing activity was found to be mostly gB mediated since adsorption of sera with recombinant gB reduced the fibroblast neutralizing titer of naturally seropositive sera two-fold (no adsorption 1:11; adsorption with 15 µg gB 1:6). In contrast, following recombinant gB adsorption of naturally seropositive sera the epithelial

entry neutralizing titer did not change (no adsorption 1:1418; adsorption with 15 μg gB 1:2183). Adsorption of a single gB/MF59 recipient serum with recombinant gB removed both fibroblast and epithelial entry neutralizing activities (fibroblast: no adsorption 1:5, 20 μg gB 1:2; epithelial: no adsorption 1:40, 20 μg gB 1:14). [62]

Endothelial entry neutralizing activities of naturally seropositive sera are approximately 128-fold higher than fibroblast and two-fold higher than epithelial entry neutralizing titers. Neutralizing activity in endothelial cells is detectable earlier post infection than fibroblast entry neutralizing activity. Sera from 18 pregnant women were tested for neutralizing activity in fibroblast and endothelial cells at 10, 30, 60, 90, 180, and 360 days post primary infection. Fibroblast entry neutralizing activity was not detectable until 30-60 days post infection and peaked at a titer of 1:16 at 30 days post infection. Endothelial entry neutralizing activity was detectable as early as 10 days post infection and peaked at 1:546 at 90 days post infection. Neutralizing activities in endothelial and epithelial cells is similar for virus VR1814, which contains functional UL128-131 genes, and AD169 with a repaired *UL131* gene. The neutralizing activities of sera against AD169 and *UL131* repaired AD169 are similar in fibroblast cells.[63] These data suggest that antibodies to UL128-131 play a role in the higher endothelial and epithelial entry neutralizing activities compared to fibroblast entry neutralizing activity following natural infection but not vaccination with Towne or gB/MF59. This supports the development of vaccines that target the epithelial entry mediators UL128-131, a feature lacking in current vaccines.

Rationale for this research

Congenital CMV disease is a serious health issue that causes neurologic sequelae in a large number of young children each year. Vaccine development over the past 40 years has

focused on induction of systemic gB antibodies and pp65 T cell responses. The most promising vaccine to date is the gB/MF59 protein subunit vaccine that provides protection against CMV infection in about half of the women of childbearing age tested in a phase II trial. This level of protection may be sufficient for herd immunity but is not ideal. The gB/MF59 vaccine provides proof of concept that a subunit vaccine against CMV is possible but this vaccine needs to be improved upon before broad use for protection against CMV.

Until recently CMV vaccines were evaluated by measuring neutralizing antibodies on fibroblast cells. New research demonstrating that neutralizing antibodies following natural infection are 48-fold higher when measured on epithelial cells[62] suggests that CMV vaccine efficacy should involve evaluation on both fibroblast and epithelial cells. This research also opens up the possibility of new subunit vaccine targets. CMV proteins UL128, UL130, and UL131 are required for infection of epithelial but not fibroblast cells. Neutralizing antibodies to these proteins have been identified following natural infection. In order to be expressed on the virion envelope, functional genes for all three proteins must be present in the CMV genome. Live attenuated vaccines and laboratory strains of CMV all have a mutation in one of these genes, and therefore do not express the complex on their virion envelope. Importantly, the Towne and gB/MF59 vaccines do not induce epithelial neutralizing activities comparable to natural infection since they lack epithelial entry mediator targets.

Vaccines targeting epithelial entry mediators, such as UL128, UL130, and UL131, may also improve mucosal immunity. Since CMV infection begins at mucosal surfaces, induction of mucosal immunity by vaccines may be necessary for protection. The gB/MF59 vaccine induces measurable gB antibodies in the saliva of about half of vaccinated adults but neutralizing activity of these antibodies was not determined. Since this vaccine is about 50% protective against CMV,

levels of salivary gB-specific antibodies may correlate with protection. The mucosal immune response to CMV in naturally infected and vaccinated individuals is poorly characterized. A better understanding of natural mucosal immunity, particularly of saliva, will aid in vaccine development by providing new goals for vaccine immunogenicity and potential correlates to protection.

This study improved our understanding of CMV mucosal immunity by characterizing the neutralizing activity of saliva from naturally infected individuals. These studies support the use of saliva in evaluating future CMV vaccines. Since current vaccines do not target epithelial entry mediator targets that will induce high levels of salivary epithelial entry neutralizing activity, vaccine approaches targeting these proteins were explored. Rabbits were inoculated with peptides from UL128, UL130, and UL131 to evaluate the potential of a peptide-based vaccine targeting epithelial entry. To address the immunogenicity of UL128, UL130, and UL131 proteins, both individually and in combination, mice were vaccinated with DNA constructs expressing each protein. The studies presented here demonstrate the potential of epithelial entry mediators as vaccine candidates and the importance of evaluating epithelial entry neutralizing activity in vaccinated subjects.

MATERIALS AND METHODS

Study populations and sample collection

Serum and saliva samples were obtained from mothers of children at the Virginia Commonwealth University Medical Center daycare and non-daycare associated adults from the University community. Serum and saliva samples from Towne vaccine recipients (obtained 2-9 months post immunization), salivas from daycare children under two, and salivas from adolescents were obtained during previous studies.[50,79,95,96] The appendix contains detailed information on populations studied. Serum and/or saliva samples were screened for evidence of CMV antibodies using an enzyme-linked immunosorbent assay (ELISA). Informed consent was obtained from all subjects or their guardians and protocols were approved by the Virginia Commonwealth University Committee for the Conduct of Human Research.

ELISA

Serum

Adult sera were assayed for CMV seropositivity by gB-ELISA.[51] Immulon I plates (Dynatech laboratories, Chantilly, VA) were coated with 100 µl per well recombinant gB (Chiron) diluted to 10 µg/mL in carbonate buffer (pH 9.6) (antigen wells) or 100 µl per well of

carbonate buffer alone (control wells) and incubated overnight at 4°C. Plates were then blocked at room temperature for one hour with 0.05% Tween-20 (PBST) with 3% bovine serum albumin (BSA). Sera were diluted 1:100 and then four-fold serially diluted in phosphate buffered saline with PBST with 3% BSA. Starting with the 1:1600 dilution, 100 µl of diluted samples were added to duplicate wells either coated with gB or control buffer. Plates were incubated for 90 minutes at 37 °C in a 5% CO_2 atmosphere then washed four times with PBST. 100 µl of anti-human IgG conjugated to alkaline phosphatase (Tago, Burlingame, CA) diluted 1:500 in PBST with 3% BSA was added to the plate. Plates were incubated for 60 min at 37 °C in a 5% CO_2 atmosphere then washed four times with PBST. The assay was developed with 100 µl of *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) at 1 mg/mL in 10% diethanolamine buffer (pH 9.6) and incubated at room temperature until strong color developed in control CMV positive serum wells (30-60 minutes). Plates were read in a spectrophotometer at 405/490 nm. O.D. from the control well was subtracted from O.D. in the gB-coated well to determine the ΔO.D. value for each sample. The gB titer was determined by the highest dilution with a ΔO.D > 0.1. A seropositive serum known to have a gB-ELISA titer of 1:64,000 was used as a control.

Saliva

Children and adolescents were evaluated for CMV positivity by detection of gB-specific IgG in saliva as described.[50,51] The saliva protocol was similar to the serum protocol (above) except that plates were coated with 5 µg/mL or 10 µg/mL recombinant gB, saliva serial dilutions started at 1:4, and the anti-human IgG was diluted 1:250.

Avidity

Avidity was determined using a commercial kit (Radim, Florence Italy) per the manufacturers instructions. Briefly, sera were diluted 1:300 in sample buffer, added in duplicate to wells of the ELISA plate included in the kit, and incubated for 1 hour at 37 °C in a 5% CO_2 atmosphere, then washed four times with wash buffer (included in the kit). An equal volume of dissociation buffer, which contains urea, was added to one duplicate well. Sample buffer was added to the other. The plate was then incubated at 37 °C in a 5% CO_2 atmosphere for 30 minutes then washed four times with wash buffer. Anti-human IgG conjugated with horseradish peroxidase was added for 30 minutes at 37 °C in a 5% CO_2 atmosphere then washed four times with wash buffer. The assay was developed with a tetramethylbenzidine solution for 10 minutes then stopped by addition of blocking reagent. O.D. was read in a spectrophotometer with 450/620 nm and 405 nm. Percent avidity was calculated by dividing the O.D. with dissociation buffer by the O.D. with sample buffer then multiplying by 100. Percent avidity for samples with O.D.>3000 in the 450/620 nm read are calculated based on the 405 nm O.D.

Cells and virus

Cells

Table 10 summarizes the cell lines used. MRC-5 (ATCC CCL-171), ARPE-19 (ATCC CRL-2302), and HBE4-E6/E7 (ATCC CRL-2078) cells were obtained from ATCC. HFK-2, Cx, V428, and HTE 21505 were a kind gift from Aloysius Klingelhutz. They were derived and immortalized by retroviral transduction of human papilloma virus-16 E6E7 as previously described.[97] BeWo clone b30 (a kind gift from Phillip Gerk) are a human choriocarcinoma monolayer-forming clone originally derived from a BeWo ATCC stock (ATCC CCL-98).[98]

ARPE-19 cells were propagated in high glucose Dulbecco's modified Eagle medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/L penicillin, 10 mg/L streptomycin (Gibco-BRL) (DMEM). MRC5 cells were propagated in modified Eagle medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/L penicillin, 10 mg/L streptomycin (Gibco-BRL) (MEM). HFK-2, Cx, V428, and HTE 21505 cells were propagated in keratinocyte serum free medium (KSFM, GIBCO 17005042) supplemented with 5 ng/mL human recombinant epidermal growth factor 1-53 (Invitrogen) and 0.05 mg/mL bovine pituitary extract (Invitrogen). HBE4-E6/E7 cells were propagated with KSFM supplemented with 5 ng/ml human recombinant epidermal growth factor 1-53, 0.05 mg/ml bovine pituitary extract, and 10 ng/ml cholera toxin (Sigma). BeWo cells were grown in low glucose Dulbecco's modified Eagle medium without sodium bicarbonate (CellGro), supplemented with 10% fetal calf serum, 10,000 IU/L penicillin, 10 mg/L streptomycin (Gibco-BRL), and MEM nonessential amino acids (Gibco-BRL). HEK-293T cells, a kind gift from Deborah Parris, were propagated in low glucose Dulbecco's modified Eagle medium (CellGro) supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/L penicillin, 10 mg/L streptomycin (Gibco-BRL) (DMEM-LG). All cell cultures were maintained at 37 °C in a 5% CO_2 atmosphere.

Table 10: Cell lines

Cell line		*Tissue (cell type)*
MRC-5	fibroblast	fetal lung
ARPE-19	epithelial	retinal pigment epithelium
HEK-293T		kidney
HFK-2		foreskin (keratinocyte)
Cx		cervix (keratinocyte)
V428		vagina
HTE 21505		tonsil
HBE4-E6/E7		bronchus
BeWo		choriocarcinoma

Viruses

Virus HB15-t178b was derived from bacterial artificial chromosome (BAC) clone HB15Tn7Δk,[99] which contains the CMV strain AD169 genome,[100] by transposition of a green fluorescent protein (GFP) reporter cassette into the *att*Tn7 site, as described.[101] Virus HB15-t178b retains a *UL131* frame shift mutation intrinsic to strain AD169. Virus BAD*r*UL131-Y4 (a gift from Thomas Shenk and Dai Wang) was derived from a different BAC clone of the CMV strain AD169 genome[102] that was first modified to express GFP[103] and then, by repair of the *UL131* mutation, to express a functional UL131 protein.[25] BAD*r*UL131-Y4 was propagated in ARPE-19 cells. Viral stocks were prepared from cell culture media that was clarified by centrifugation, adjusted to 0.2 M sucrose, aliquoted, stored at –80 °C, and titered on MRC-5 or ARPE-19 cells by limiting-dilution in 96-well plates as described.[62]

Neutralization assay

Neutralization assays were conducted as described previously.[62,104] Briefly, samples were two-fold or ten-fold diluted in cell culture media (or in some cases, saliva), then two-fold serially diluted in cell culture media. Serially diluted samples were incubated with an equal volume of culture media containing 5000 PFU of green fluorescent protein (GFP)-tagged virus BAD*r*UL131-Y4,[25] incubated for 1 h at 37 °C, and transferred to black-walled, clear-bottom 384-well plates containing confluent MRC-5 or ARPE-19 cells. Representative images were taken three (MRC-5) or four (ARPE-19) days post infection using a Nikon Diaphoto 300 microscope. GFP fluorescence was quantitated using a Victor^{3}V 1420 multi-label counter (Perking Elmer) at day seven post infection. Fifty percent inhibitory concentration (IC_{50}) values and standard errors of the means were calculated using Prism software (GraphPad Software, Inc.) by plotting the

means of triplicate GFP values for each serum dilution against $\log_2$ serum concentration, calculating the best fit four-parameter equation for the data, and interpolating the serum dilution at the mid-point of the curve as the IC_{50} neutralizing titer.[104] To evaluate neutralization of viral entry into mucosal epithelial cells, rabbit anti-peptide sera were used at a 1:20 dilution and photomicrographs were taken seven days post infection. All samples were assayed in triplicate to obtain geometric mean IC_{50} values +/- standard errors.

Entry assay

Virus stocks were carefully titered using MCR-5 fibroblast cells, then matching amounts of HB15-t178b or BAD*r*UL131-Y4 were used to infect replicate cultures of confluent cells prepared in 24-well plates. After 24 h the cultures were washed three times with PBS and fresh medium was added. Photomicrographs were taken daily post infection using an Olympus LX70 Inverted UV microscope or a Nikon Diaphoto 300 microscope.

Animals

Six week old female Balb/c mice were purchased from Jackson Labs, Bar Harbor, Maine. Mice were kept in pathogen free conditions at Virginia Commonwealth University, Richmond, VA or at Explora Vivarium San Diego, CA in accordance with the institutional animal care and use committee.

Rabbit antisera

Rabbit antisera to UL128, UL130, and UL131 peptides were a gift from David Johnson and Brent Ryckman. Details of the production are given below. The amino acid sequence of each protein was evaluated using computer algorithms that predict hydrophilic, antigenic, and surface

exposed domains. From these results one peptide from each protein was selected based on empirical experience that N- or C-terminal positions, charged residues, and prolines are desirable. Peptides DQYLESVKKIHKRLDV (UL128 residues 147-162), SWSTLTANQNPSPPWSKLTY (UL130 residues 27-46), and SDFRRQNRRGGTNKRTT (UL131 residues 90-106) were synthesized with C-terminal cysteines by PeptidoGenics (Berkley, CA) and coupled to maleimide activated keyhole limpet hemocyanin (KLH) under conditions that produce conjugates in which the peptides comprise 15-30% of the mass. For each peptide one New Zealand White rabbit was immunized with 500 to 1000 µg of KLH-conjugated peptide mixed with Freund's adjuvant, then boosted three times at 4-6 week intervals with decreasing doses of KLH-conjugated peptides (250 µg, 100 µg, and 50 µg) in TiterMax Gold adjuvant (Sigma, St. Louis, MO). An isoleucine at position 10 of the UL128 peptide was unintentionally inserted. However, this does not prevent recognition of native UL128 (which lacks the isoleucine) by the UL128 antiserum. All three antisera have been extensively characterized elsewhere and shown to react specifically with UL128, UL130, or UL131 by immunoprecipitation and western blotting.[22,32]

DNA vaccine

Vaccine plasmid construction

The DNA vaccine vector VR10551 was a gift from Larry Smith. VR10551 is a DNA vaccine plasmid created by Vical Inc. (San Diego, CA) that includes a CMV immediate early (IE) promoter, 5' UTR and intron A from the CMV IE gene, a synthetic rabbit beta globin (RBG) consensus poly A signal and a kanamycin resistance cassette (kan^r).[105,106] The cDNAs encoding UL128, UL130, and UL131 (strain TR) inserted into vector pAdtet-7 were a kind gift

from Brent Ryckman and David Johnson.[32] To make pFastBac1-UL128, pFastBac1-UL130, and pFastBac1-UL131 each pAdtet-7 vector (pAdtet-7-UL128, pAdtet-7-UL130, pAdtet-7-UL131) and pFastBac1 were digested with NotI and BamHI. Then the UL128, UL130, and UL131 fragments were individually ligated into pFastBac1. The UL128, UL130, and UL131 coding sequences in pFastBac1 were confirmed by sequencing. VR10551-TR128, VR10551-TR130, and VR10551-TR131 were constructed by excising each ORF from pFastBac1-UL128, pFastBac1-UL130, and pFastBac1-UL131 with BamHI and XbaI and ligating the fragments into BamHI/AvrII-digested VR10551. Schematics of VR10551-TRUL128, VR10551-TRUL130, and VR10551-TR131 can be found in figure 7. DNA for the first DNA vaccine study was prepared using an Endo-free plasmid Giga kit (Qiagen) per manufacturer's instructions. Codon-optimized plasmid expression vectors encoding UL128 (VR10551-Towne128), UL130 (VR10551-Towne130), or UL131 (VR10551-Towne131) proteins (strain Towne) in DNA vaccine vector VR10551 (Vical Inc.) were synthesized by Blue Heron Biotechnology, Inc for use in the second DNA vaccine study.

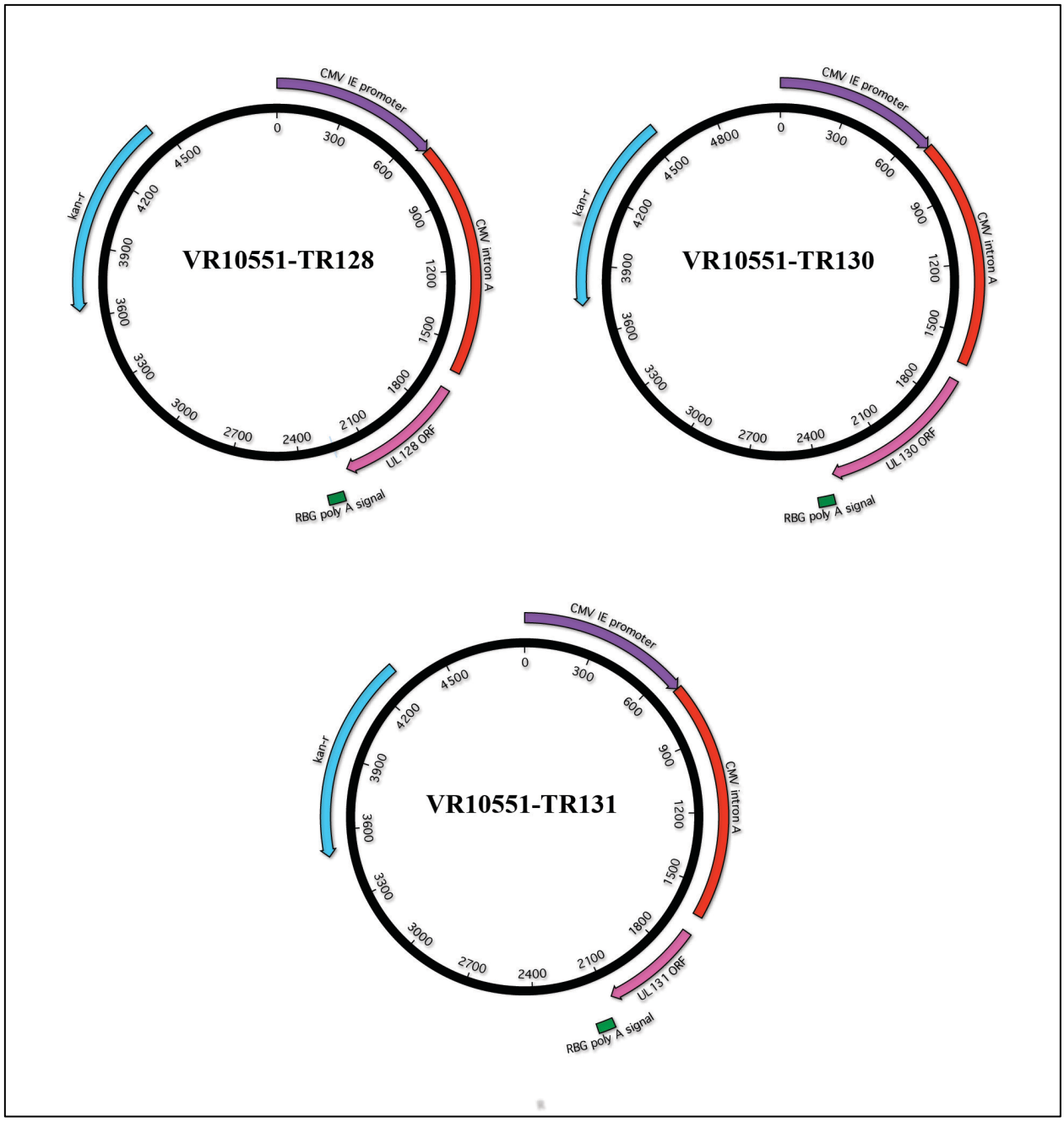


Figure 7: DNA vaccine plasmid constructs. VR10551 contains a kanamycin resistance cassette, CMV IE promoter, CMV intron A, and RBG poly A signal.

In vitro expression of vaccine plasmids

HEK-293T cells were transfected with DNA vaccine plasmids using Effectene (Quiagen) according to the manufacturer's instructions. GFP expression plasmid pMA178b was used as a control.[107] Cells were plated in 6-well plates 48 hours prior to transfection. For each well 4 µg of DNA was diluted in 100 µl of buffer EC, mixed with 3.2 µl of enhancer, vortexed, then incubated for 5 min. at room temperature. 10 µl of Effectene was added, the mixture was vortexed, then incubated for 10 min. at room temperature. 600 µl of DMEM-LG was then added to the DNA complexes. 1500 µl of fresh DMEM-LG was added to each well, then 600 µl of the DNA/Effectene reaction was added drop wise. Transfected cells were incubated at 37 °C in a 5% CO_2 atmosphere for 42-72 hours. Cells were harvested into 50 mL conical tubes using enzyme free dissociation buffer (Gibco-BRL) and washed two times with PBS-EDTA by centrifugation at 2000 revolutions per minute (RPM). Cells were lysed in 0.5% NP40 in PBS-EDTA for 10 minutes. Lysates were clarified by centrifugation at 2,500 RPM for 10 minutes at room temperature. Supernatants were aliquoted and stored at -20°C.

Mouse Immunizations

Mice were immunized and bled at Virginia Commonwealth University for the first DNA vaccine study and at Explora Vivarium (San Diego, CA) by Vical, Inc. employees for the second DNA vaccine study. Female Balb/c mice were immunized with 90 µg of DNA formulated with Vaxfectin® per leg in the rectus femoris muscle three times, three weeks apart. To formulate DNA with Vaxfectin® (lot# PRO-09-013), a vial containing 2.18 mg of Vaxfectin® at room temperature was reconstituted in 1mL of 0.9% saline by vortexing for 5 minutes (properly reconstituted Vaxfectin® appears milky with no aggregates). DNA at 2 mg/mL in 0.9% saline

20mM sodium phosphate buffer was placed in the bottom of a round bottom tube and an equal volume of the reconstituted Vaxfectin® was slowly added with a $26G^{5/8}$ needle. The mixture was then inverted 5 times to mix, resulting in a milky solution. The DNA/Vaxfectin® mixture was then diluted with 0.9% saline in 20 mM sodium phosphate. 50 µl of the formulated DNA mixture was injected into the rectus femoris muscle bilaterally using a $28G^{1/2}$ needle. Mice were bled using a 5 mm Goldenrod lancet to pierce the facial vein prior to each immunization. Blood was collected in gold top gel serum separator tubes (BD). Samples were kept at room temperature for 20-60 minutes then spun in a microfuge at 1200 RPM for 5 minutes at room temperature. The serum in the top layer was transferred to a 2 mL screw cap tube and stored at -20°C. Three weeks following the last immunization, mice were euthanized and blood was collected via cardiac puncture. Blood was collected in gold top gel serum separator tubes and processed as described above.

Adenovirus expression of UL128, UL130, and UL131

Adenovirus type 5 (Ad5) vectors expressing tet, gH, gL, UL128, UL130, or UL131 (CMV strain TR) were a kind gift from Brent Ryckman and David Johnson.[32] Genes expressed in this system are under control of a tetracycline inducible promoter and therefore require co-infection with Ad5-tet for transgene expression.[108] Ad5 vectors lack E1 so they must be propagated in HEK-293T cells that express E1. Transgene expression occurs in cell lines with or without E1.

Virus stocks were grown in HEK-293T cells infected at a MOI of 5 and incubated at 37°C in a 5% CO_2 atmosphere until the cells were falling off the flask. Culture media were harvested at 72 h and clarified by centrifugation at 2000 RPM for 10 minutes twice. Virus stocks

were aliquoted and stored at -80°C. Stocks were titrated in 96-well plates on HEK-293T cells. Titers were determined based on the number of wells with cytopathic effect 4-5 days post infection.

HEK-293T cells were infected with each of the Ad5 vectors at a MOI of 100 plus Ad5-tet at a MOI of 20. Cells were harvested into 50 mL conical tubes using enzyme free dissociation buffer (Gibco-BRL) and washed two times with PBS-EDTA. Cells were lysed in 0.5% NP40 in PBS-EDTA for 10 minutes. Lysates were clarified at 2,500 RPM for 10 minutes at room temperature. Clarified lysates were aliquoted and stored at -20°C.

Western blot

Human or mouse serum antibodies to UL128, UL130, or UL131 were assayed by standard western blot methods[32] using transfected HEK-293T cells or Ad5 infected HEK-293T cells as antigens prepared as described above. Cell lysates were mixed 1:1 with Laemeli sample buffer (Biorad), heated to 85°C for five minutes, and cooled to room temperature for five minutes. Samples were then loaded onto 10-20% Tris-HCL gels (Biorad) and separated by SDS-PAGE in Tris/Glycine/SDS buffer (Biorad). Gels were transferred to 0.2 µm nitrocellulose membranes (PALL Life Sciences) in Dunn carbonate buffer (100 mM $NaHCO_3$ 30 mM Na_2CO_3 in dH_2O pH 9.9) for 2.5-3 hours at 0.6 mA as described,[32] then membranes were blocked in 5% milk PBST for one hour. Membranes were incubated overnight at 4 °C with rabbit sera specific for UL128 (5 µl), UL130 (0.25 µl), or UL131 (1 µl),[32] 1 µl human sera, or 10 µl mouse sera diluted in 5% milk PBST. Blots were washed for 20 min. three times in PBST. Reactivities were detected using HRP-conjugated goat anti-rabbit or anti-human IgG (Thermo), as appropriate,

washed for one hour 3-4 times in PBST, followed by West Pico SuperSignal chemiluminescent substrate (Pierce) and exposure of X-ray film.

Statistics

Non-linear regression, linear regression, students paired t-test, Fischer's exact test, and Pearson's correlation were performed with Prism 5.

NEUTRALIZING ACTIVITY OF SALIVA AGAINST CMV

Anti-CMV activity of saliva is poorly characterized

CMV transmission occurs at mucosal surfaces so immunity in these locations is vital to blocking CMV infection. Women with children in daycare frequently acquire CMV from their children;[3] presumably in this population transmission occurs via the oral cavity. Saliva provides mucosal immunity in the oral cavity with a combination of sIgA, IgG, IgA, and other immune factors.[46] In seropositive individuals salivary neutralizing antibodies most likely prevent reinfection with new CMV strains by neutralizing virus at the initial inoculation site. Vaccination of seronegative individuals should induce salivary neutralizing activity to prevent primary infection via the oral cavity to mimic the mucosal protection afforded by saliva in seropositives.

Neutralizing activity of seropositive serum is much higher on epithelial cells compared to fibroblast cells[62] and this observation is expected to also be true for salivary neutralizing activity. Differences in neutralizing activity between cell types can be attributed to CMV's requirement for different virion proteins for entry into each cell type. Entry of CMV into epithelial and endothelial cells requires gH/gL/UL128-131.[20,26,29,33] Antibodies specific for this complex will

not block entry into fibroblast cells so it is assumed that at least some of the difference between epithelial and fibroblast neutralizing titers can be attributed to antibodies specific for the gH/gL/UL128-131 complex. The anti-CMV neutralizing activity of saliva is not well studied. Since mucosal sites are predominately epithelial cells neutralizing activity found in the saliva is expected to include antibodies specific for epithelial entry meditators such as UL128, UL130, and UL131 because fibroblast entry targets, such as gB, do not have potent epithelial entry neutralizing activity.[62]

Antibodies against CMV in whole saliva occur at concentrations approximately 1,000-fold below those in serum for both IgG and IgA.[50,51] This is probably due to the fact that significant portions of the antibodies in whole saliva are passively transferred from the serum. Gingival crevicular fluid, in contrast, contains a higher proportion of CMV specific IgA compared to IgG due to local IgA production.[109] Since CMV commonly causes initial infection in the oral mucosa, these CMV-specific salivary antibodies may be important for protection against subsequent CMV infection. Unfortunately, the anti-CMV antibody response in the saliva is poorly characterized.

The majority of the anti-CMV antibody response in the serum is to gB.[5] Levels of salivary gB-specific IgG were determined in children under three years old shedding CMV in their urine or saliva. Unstimulated saliva was collected from 58 children (20 CMV positive, 38 CMV negative), and screened for gB-specific IgG by ELISA. All CMV positive children had positive gB-specific IgG ELISA results while none of the CMV negative children tested positive for gB in this assay.[50] gB-specific antibody levels in the saliva of adults has also been studied. All naturally infected adults and gB/MF59-vaccinated adults tested had detectable gB-specific

IgG in their saliva. Towne vaccine recipients had a lower occurrence of gB-specific IgG in their saliva (42%). Approximately half of the naturally infected and gB/MF59-vaccinated adults but none of the Towne-vaccinated adults had detectable gB-specific sIgA in their saliva. 70% of gB/MF59 vaccinated adults but no Towne-vaccinated or naturally infected adults had detectable gB-specific IgA in their saliva.[51] These data suggest that natural immunity to CMV in adults induces IgG and sIgA antibodies in the saliva that may provide protection against secondary infection.

Only one report of salivary CMV neutralizing antibodies is published. In this study unstimulated saliva was collected from 54 CMV positive and 11 CMV negative infants in Sapporo, Japan in 1980. Neutralizing titers were determined based on 60% plaque reduction with the Davis strain of CMV on human embryonic lung fibroblasts. Saliva from CMV negative infants did not neutralize virus in this assay. Saliva from 76% of infants tested neutralized virus infection above the background titer of 1:4. In seven infants shedding CMV in their urine and saliva, neutralizing titers were higher in serum than saliva but the level of neutralizing antibodies in the saliva did not change once the child stopped shedding CMV. Analysis of pooled saliva samples from all infants with salivary fibroblast neutralizing activity demonstrated that sIgA makes up a large proportion of the observed neutralizing activity. These data suggest that the amount of locally produced CMV-specific sIgA is not sufficient to neutralize virus shed in the saliva of infants because the presence of sIgA at levels capable of neutralizing infection *in vitro* does not influence shedding of CMV in the saliva. It is interesting that saliva of these infants neutralized *in vitro* but not *in vivo,* the authors of this paper suggest that this phenomenon is due to infectious virus-antibody complexes in the saliva.[110]

CMV infection of epithelial cells requires the entry mediators UL128, UL130, and UL131 on the virion surface. Antibodies to one or more of these proteins are present in the serum[52] and may be present in the saliva. Presence of gB specific IgG and sIgA in the saliva of naturally infected individuals suggests that saliva may have anti-CMV activity. Due to the mucosal nature of the oral cavity, antibodies other than gB are most likely important because gB antibodies alone do not provide the epithelial entry neutralizing activity seen following natural infection. Neutralizing activity of saliva from naturally infected adults has not been studied. Therefore, saliva from naturally infected adults, Towne-vaccinated adults, adolescents, and young children were tested for neutralizing activity on fibroblast and epithelial cells.

Results

Saliva has no intrinsic anti-CMV activity

To ascertain if human saliva contains non-specific CMV neutralizing factors, salivas from 18 CMV seronegative adults were tested (n=12 daycare associated adults; n=4 non daycare associated adults; n=2 placebo recipients from a Towne vaccine trial, see appendix). None had CMV neutralizing activities, regardless of cell type, at dilutions as low as 1:2 (for representative data see Fig 8). To determine if saliva contains factors that inhibit neutralizing antibodies, epithelial entry neutralizing activities were measured for a seropositive serum diluted 1:10 with culture medium or with salivas from two seronegative adults. The IC_{50} for the serum diluted in culture medium (1:1986) was no different from the IC_{50}s for the serum diluted in either saliva (1:2011 and 1:2022). Representative data for one saliva are shown in Fig. 9. These results demonstrate that in the absence of CMV-specific antibodies, saliva contains no factors that non-

specifically inactivate virus. Moreover, that serum neutralizing activity was unaffected by saliva suggests that saliva lacks factors that impair antibody neutralizing activity.

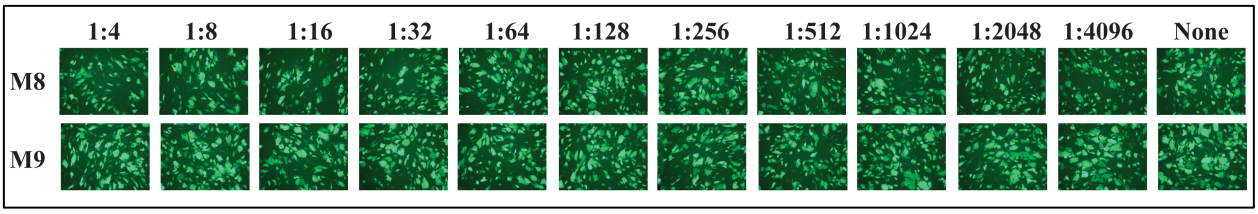


Figure 8: Salivas from CMV negative adults do not neutralize CMV. Saliva samples from two CMV seronegative adults were serially diluted, mixed with 5000 PFU of Bad*r*UL131 for 1 hour then added to epithelial cells. Images were taken at four days post infection. Saliva dilutions are indicated above the images.

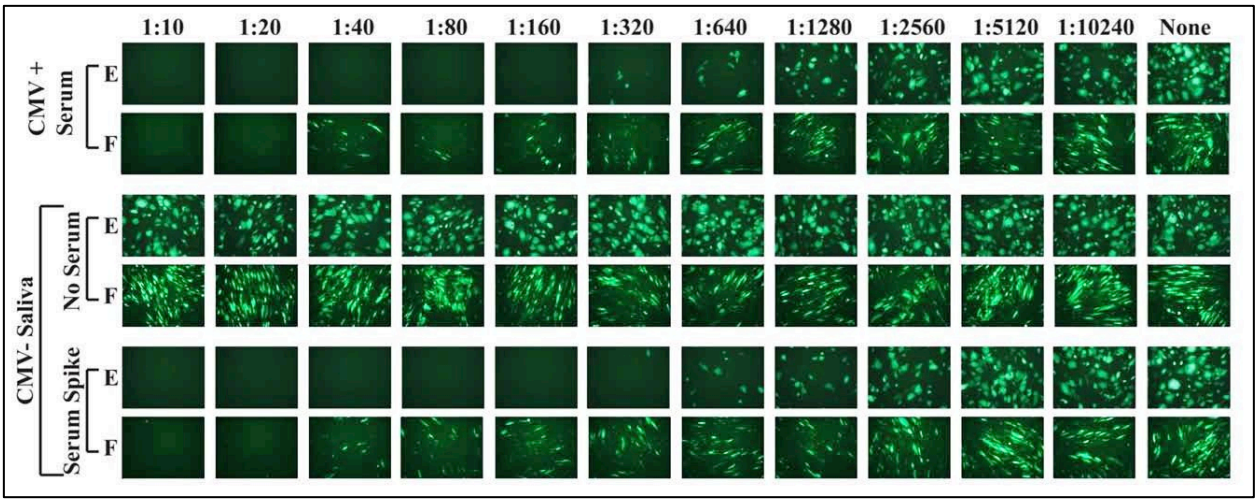


Figure 9: Saliva has no intrinsic anti-CMV activity. Serum and saliva samples were serially diluted, mixed with 5000 PFU of Bad*r*UL131 for 1 hour then added to epithelial (E) or fibroblast (F) cells. Images were taken at three (fibroblast) or four (epithelial) days post infection. Serum from a seropositive adult serially diluted in media is shown in the top panel. The middle panel shows saliva from a seronegative adult. The bottom panel shows the seropostivie serum from the top panel diluted in the saliva from the middle panel.

Saliva from some seropositive individuals neutralizes CMV infection

Children under two years of age

Frozen saliva samples from a previous study[96] of 17 children attending large group daycare were screened for neutralizing activity on epithelial cells (demographics can be found in the appendix). Prior to freezing all saliva samples in this group tested positive for gB-specific IgG by ELISA, confirming that these subjects were CMV infected. Ages ranged from 2-24 months. Saliva was not cultured at the time of collection to determine if the children were shedding CMV in their saliva, but 7 of the 17 children were cultured 3-4 weeks following saliva collection and were positive for CMV in the urine. None of the 17 young children tested had detectable neutralizing activity in their saliva, even though they all had gB-specific IgG in their salivas.

Adolescents

Saliva from eight adolescents attending a STD clinic obtained from a previous study (see appendix),[95] were screened for neutralizing activity. Prior to freezing all of the samples tested positive for gB-specific IgG by ELISA. At the time of collection saliva was not screened by CMV culture. No other body fluids, such as urine or serum, were collected from these individuals. Similar to the young children described above, no neutralizing activity was detectable in salivas positive for gB-specific IgG by ELISA in adolescents attending a STD clinic.

Adults

All of the 16 seropositive adults lacked detectable salivary neutralizing activity using fibroblasts, but salivas from eight adults neutralized epithelial cell entry with IC_{50} titers ranging

from 1:6 to 1:28 (see representative data, Fig 10 and Table 11). Seven of the adults were mothers of children in daycare, while the rest were non-daycare associated adults. Salivary neutralizing activity was more frequent among daycare mothers than non-daycare-associated adults (6/7 vs. 2/9, Fischer's exact test, p=0.04). Age of the child in daycare did not correlate with saliva titers of the mothers tested (linear regression, r = 0.03370, p = 0.9369, R^2 = 0.001136). Immunological data for the adult subjects are summarized in Table 11.

To determine if the presence of salivary neutralizing activity corresponds to recent infection, the avidities of CMV-specific serum IgG in the adult group were determined. The three subjects with high salivary neutralizing activity had high serum IgG avidity, suggesting that these subjects were not infected recently; however that three subjects with low avidity also had salivary neutralizing activity (A4, M1, and M3; Table 11) suggests that salivary neutralizing activity can occur shortly after infection. Within the adult group mean serum titers for subjects with salivary activity were statistically higher than for those without (Student's *t* test, p = 0.04, r^2 = 0.5) and a linear correlation was observed between serum and salivary neutralizing titers (linear regression, r = 0.7, p = 0.005, R^2 = 0.4, Figure 11).

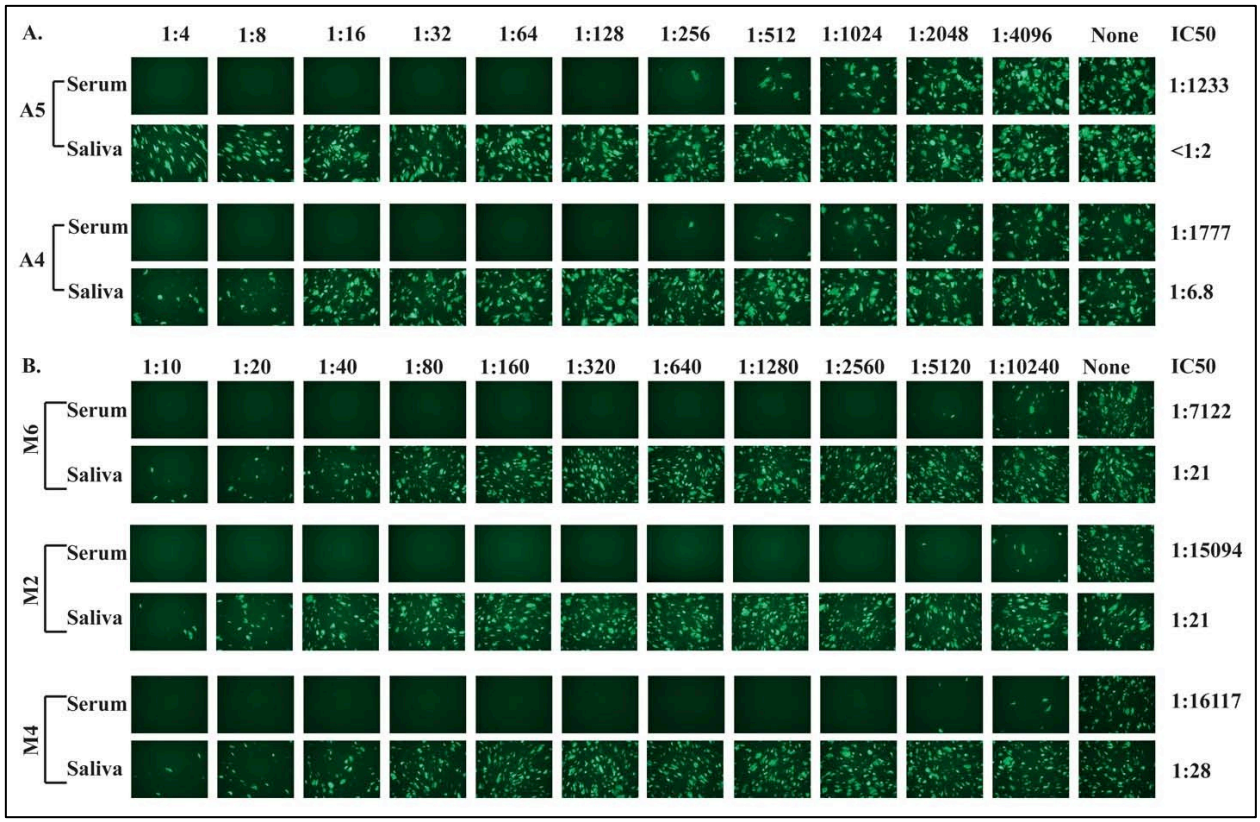


Figure 10: Representative epithelial entry neutralizing assays of sera and salivas from naturally CMV infected adults. Serum and saliva samples from naturally infected adults were serially diluted, mixed with 5000 PFU of Bad*r*UL131 for 1 hour then added to epithelial cells. Images were taken at four days post infection. GFP fluorescence was quantitated at seven days post infection using a Victor^{3}V 1420 multi-label counter (Perkin Elmer). IC50 values, shown to the right of the images, were calculated using the non-linear regression function in Prism 5 (GraphPad Software, Inc.) as described in the Materials and Methods section. (A) Two subjects without children in daycare. (B) Three mothers of children in daycare.

Table 11: Immunologic Characteristics of the Adult Study Population

	Serum			Epithelial Neutralization				Fibroblast Neutralization	
				Serum		Saliva		Serum	
Subject[a]	gB-ELISA	avidity[b]	UL130[c]	IC_{50}[d]	range[e]	IC_{50}[d]	range[e,f]	IC_{50}[d]	range[e]
A1	102400	37	-	1124	962-1314	< 2	NA	186	122-285
A2	6400	47	-	303	223-393	< 2	NA	67	33-136
A3	25600	46	-	534	441-648	< 2	NA	178	105-301
A4	102400	33	-	1777	1578-2004	6.8	6-8	453	341-602
A5	25600	41	-	1233	1081-1407	< 2	NA	256	105-629
A6	25600	49	-	3686	3405-3998	< 2	NA	73	51-104
A7	25600	52	-	5031	4401-5760	< 2	NA	254	207-313
A8	25600	49	+	3630	3307-3986	5.7	5-6	101	88-117
A9	102400	35	-	3072	2742-3435	< 2	NA	188	163-217
M1	25600	31	-	2261	1863-2740	10.0	5-18	237	210-267
M2	256200	51	+	15094	13233-17232	21.4	15-30	835	568-1230
M3	102400	21	-	3139	2642-3733	13.2	4-39	253	215-298
M4	256200	56	+	16117	14861-17456	27.9	25-32	364	323-410
M5	25600	38	-	1808	1371-2382	8.8	3-28	39	30-51
M6	102400	45	+	7122	5985-8491	21.0	18-24	150	101-222
M7	25600	39	-	181	151-218	< 2	NA	52	25-111

[a] subjects A1-9 are non-daycare associated adults; subjects M1-7 are mothers of children in daycare
[b] <35 low; 35-45 mid; >45 high
[c] determined by immunoblot
[d] reciprocal titers
[e] ranges are inverse logs of the mean log(IC_{50}) +/- standard error of the mean log(IC_{50})
[f] NA, not applicable

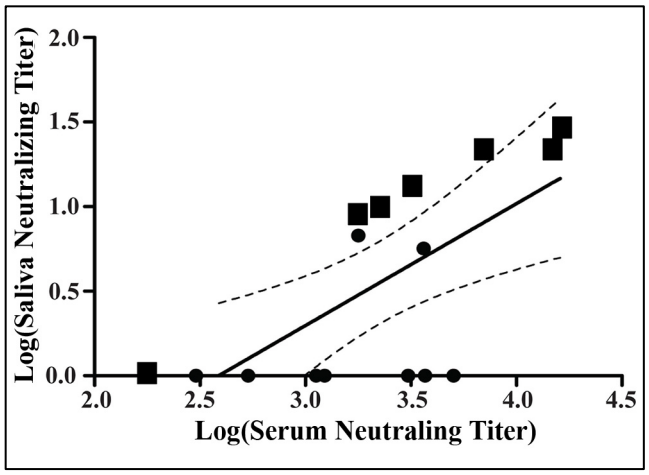


Figure 11: Saliva neutralizing titers correlate with serum neutralizing titers. Salivary and serum neutralizing titers from 16 CMV seropositive adult subjects were compared using linear regression analysis ($r = 0.7$, $p = 0.005$, $R^2 = 0.4$). Squares symbolize daycare associated subjects and circles symbolize non-daycare associated subjects. Dashed lines indicate 95% confidence limits.

Towne vaccine recipients

A sample of eight adults that had been vaccinated with 6000 PFU of the Towne vaccine all had gB-specific IgG in their serum 2-36 months post vaccination (ELISA titers 1:6400 to 1:25600, see appendix). None of the eight saliva samples tested were CMV culture positive at the time of collection. Frozen saliva samples from the same eight individuals did not have any detectable neutralizing activity on fibroblast or epithelial cells. The lack of salivary activity may be due to lower serum anti-CMV antibodies following Towne vaccination compared to natural infection (average gB ELISA for Towne recipients (appendix): 1:1840 vs average gB ELISA for naturally infected adults (Table 11): 1:77225; p=0.028).

Description of highly immune individuals

Immunologic characteristics

Three subjects (M2, M4, and M6; shaded in Table 11) had high salivary neutralizing titers (>1:20) and exceptionally high serum epithelial entry neutralizing titers (1:15100, 1:16100, and 1:7100, compared to a mean titer of 1:2070 and range of 1:1000 to 1:3000 for seropositives from our previous study.[104] In addition, when these three individuals are excluded from the present adult group the mean titer is 1:1900 with a range of 1:180 to 1:5030. Representative serum and saliva neutralizing assays for these three subjects are shown in Figure 10B and summarized in Table 11. These individuals were all mothers of young children in daycare (ages 4-32 months), and one individual (M6) has two young children (4 and 32 months). The children of these women were not screened for evidence of CMV infection or for shedding in the urine or saliva. High serum anti-CMV avidity values indicate that these women were not experiencing a recent

primary CMV infection at the time of sample collection (Table 11). However, avidity values do not provide evidence of newly acquired secondary CMV infection.

UL130 antibody detection

Although human epithelial entry neutralizing responses are believed to target the gH/gL/UL128-131 complex, western blot detection of antibodies to UL128, UL130, or UL131 in sera from naturally infected subjects has not been reported. To detect serum antibodies reactive with UL128, UL130, or UL131, lysates of cells transfected with DNA vaccine plasmids engineered to express each protein were used as antigens for western blot assays. Expression of each protein was confirmed using rabbit antisera (Fig 12). For all 16 adult seropositive sera, no reactivities to UL128 or UL131 were detected (Figs 13 and 14); however, serum antibodies to UL130 were detected in the three daycare mothers with high serum and salivary neutralizing activities (M2, M4, and M6) and in subject A8, a non-daycare associated adult (Fig 13). All other adults were negative for UL130 antibodies (Figure 14).

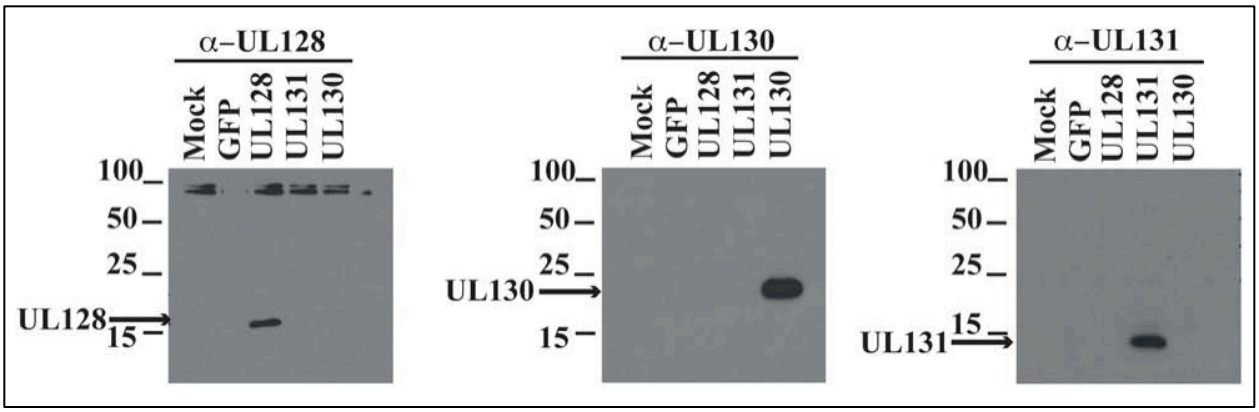


Figure 12: Transfected cell lysates express UL128, UL130, and UL131 detectable by western blot. HEK-293T cells were mock transfected or transfected with DNA vaccine plasmids (VR10551-Towne) expressing UL128, UL130, UL131, or a control GFP expressing plasmid (as indicated above each lane). Lysates were prepared at 48 hours post transfection, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were probed with anti-peptide rabbit serum to UL128, UL130, or UL131, as indicated above each blot. Migrations of molecular weight standards are shown on the left. Arrows indicate position of each protein.

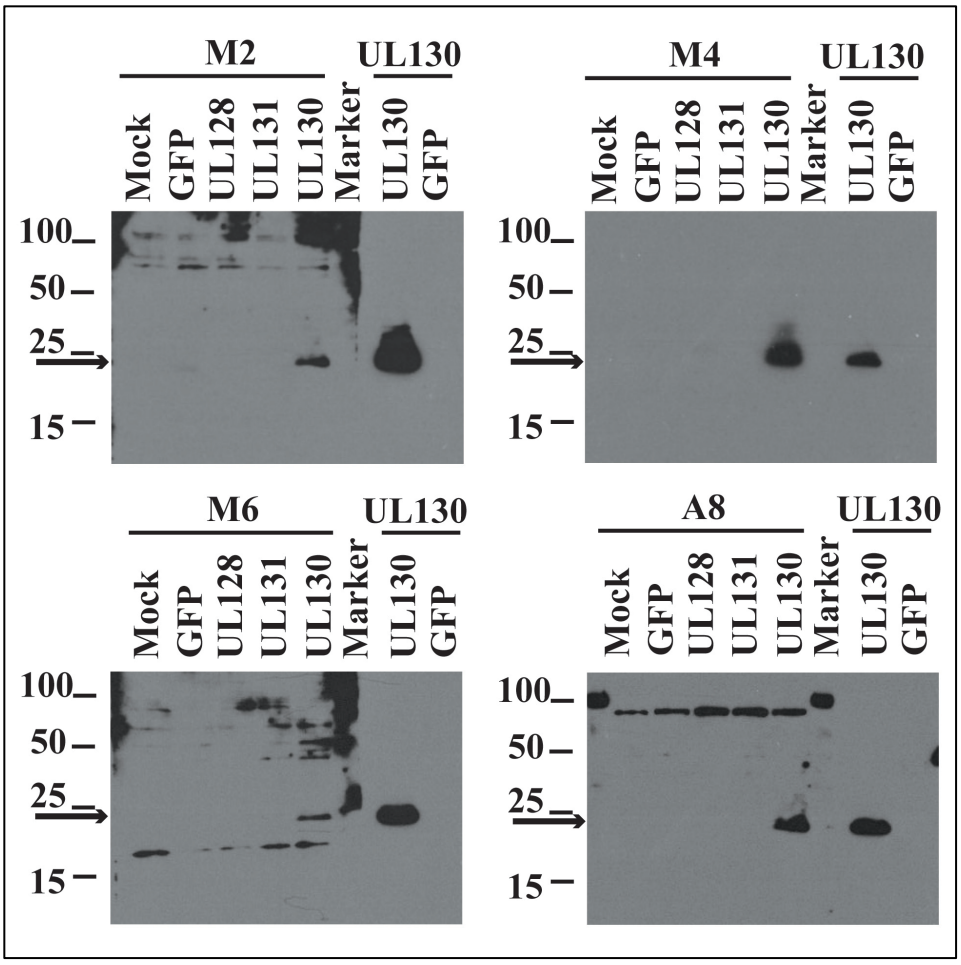


Figure 13: Adults with serum antibodies to UL130 detectable by western blot. HEK-293T cells were mock transfected, or transfected with DNA vaccine plasmids (VR10551-Towne) expressing UL128, UL130, UL131, or a control GFP expressing plasmid (as indicated above each lane). Lysates were prepared at 48 hours post transfection, proteins were separated using SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were probed with anti-peptide rabbit serum to UL130 or with sera from human subjects M2, M4, M6, or A8. Migrations of molecular weight standards are shown on the left. Arrows indicate position of UL130.

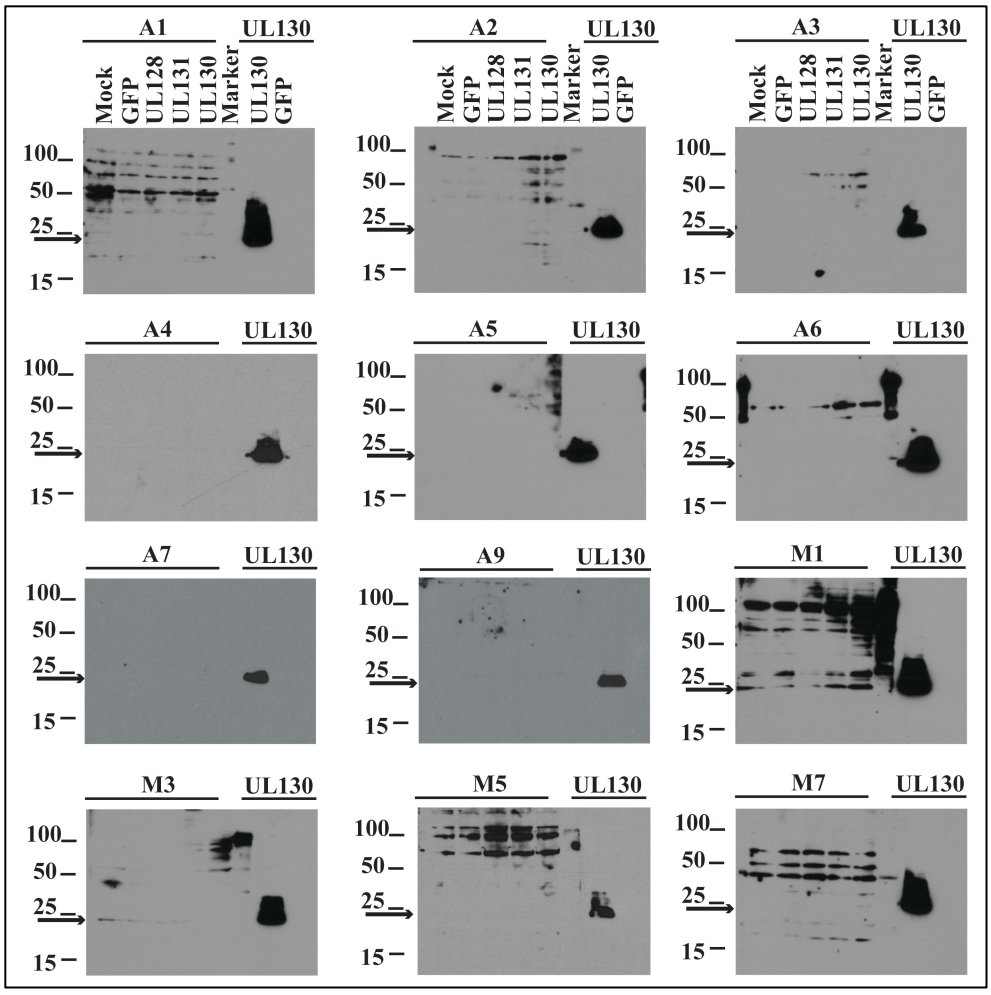


Figure 14: Adults lacking western blot-reactive UL130 antibodies. HEK-293T cells were mock transfected, or transfected with DNA vaccine plasmids (VR10551-Towne) expressing UL128, UL130, UL131, or a control GFP expressing plasmid (as indicated above each lane). Lysates were prepared at 48 hours post transfection, proteins were separated using SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were probed with anti-peptide rabbit serum to UL130 or with sera from the indicated human subjects. Migrations of molecular weight standards are shown on the left. Arrows indicate position of UL130.

Summary

Saliva from young children and adolescents did not have any detectable neutralizing activity using either fibroblast or epithelial cells. This is in contrast to a previous report of neutralizing activity on fibroblast cells of saliva from CMV infected infants.[110] Differences may be due to different age of the children tested, differences in sample collection, freezing of our samples prior to testing, or the use of different assays to measure neutralizing activity. Towne vaccine recipient saliva also did not have any detectable neutralizing activity in this assay, which may be due to the lower serum antibody levels compared to natural infection in vaccinated individuals.

Half of the naturally infected adults tested had detectable salivary neutralizing activity on epithelial cells with titers of approximately 1:6-1:28. Of these individuals, three with young children in daycare had extremely high serum neutralizing titers, the highest saliva neutralizing titers, and UL130 serum antibodies detectable by western blot. One additional individual, without children in daycare, also had salivary neutralizing activity and UL130 serum antibodies detectable by western blot. These data represent the first evidence of UL130 serum antibodies detectable by western blot. High levels of salivary neutralizing activity correlated with high serum neutralizing activity and the presence of serum antibodies to UL130.

PEPTIDES TO UL130 AND UL131 NEUTRALIZE CMV INFECTION OF MUCOSAL EPITHELIAL CELLS

Importance of epithelial cells in vaccine development

In the past CMV neutralizing activity was measured using fibroblasts as target cells. However, recent experiments demonstrate that antibodies to epitopes within the gH/gL/UL128-131 complex potently neutralize entry into endothelial, epithelial, and other cell types but have no effect on fibroblast entry.[26,30,52,63] This is because the gH/gL/UL128-131 complex is essential for entry into endothelial and epithelial cells but fully dispensable for fibroblast entry.[20,26,29] Indeed, mutations causing loss of UL128, UL130, or UL131 expression are sufficient to eliminate endothelial tropism[29] and occur within relatively few passages in fibroblasts.[24] Natural infection elicits very high titer neutralizing antibodies specific for epithelial cell entry and it has been proposed that antibodies against gH/gL/UL128-131 epitopes may comprise a significant component of this activity.[52,62,63] In contrast, epithelial entry neutralizing titers induced by the Towne live attenuated vaccine, where a mutation in the *UL130* gene prevents gH/gL/UL128-131 complex formation, or the gB subunit vaccine, were 28- and 15-fold lower, respectively, than

those induced by natural infection.[62] These results suggest that vaccine efficacy could be improved using antigens that elicit high titer epithelial entry neutralizing antibodies.

Previous studies have focused on traditional epithelial cell lines, such as ARPE-19 retinal pigment epithelium epithelial cells. Although retinal pigment epithelium cells are frequently infected *in vivo,* specifically in HIV/AIDS patients with CMV retinitis,[5] mucosal epithelial cells are most likely the first cells infected during a primary CMV infection.[3] Mucosal and secretory antibodies that neutralize viral entry into epithelial cells of the oral or genital epithelium may prevent or reduce viral transmission. For the purpose of vaccine design it is important to demonstrate that CMV infection of mucosal epithelial cells behaves *in vitro* similar to the ARPE-19 epithelial cell model. Specifically, the requirement for the gH/gL/UL128-131 complex for entry into mucosal epithelial cells is not known.

Antibodies to the gH/gL/UL128-131 complex have been identified in the serum of naturally infected adults. Identified antibodies recognized mostly conformational epitopes that required expression of multiple proteins from the complex.[52] However, it is not known if the expression of one or more of these proteins will be necessary to induce a protective immune response. Proper expression of the complex requires expression of all five genes. In the absence of UL128, UL130, or UL131, the remaining proteins are retained in the ER.[32] Immunization of animals with short peptides provides a means to evaluate the immunogenicity of linear epitopes from each protein. Rabbits were immunized with peptides generated to relatively conserved regions of UL128, UL130, or UL131 to obtain anti-peptide sera for determination of immunogenicity and the potential for a vaccine based on linear subunit epitopes. Since small peptides are not strong immunogens, each peptide was conjugated to KLH and complexed with Freund's to

increase immunogenicity for rabbit vaccination. This aggressive approach is not ideal for clinical vaccine purposes but provides cost-effective animal models to determine immunogenicity of difficult-to express proteins.

Results

CMV entry into mucosal epithelial cells requires the gH/gL/UL128-131 complex

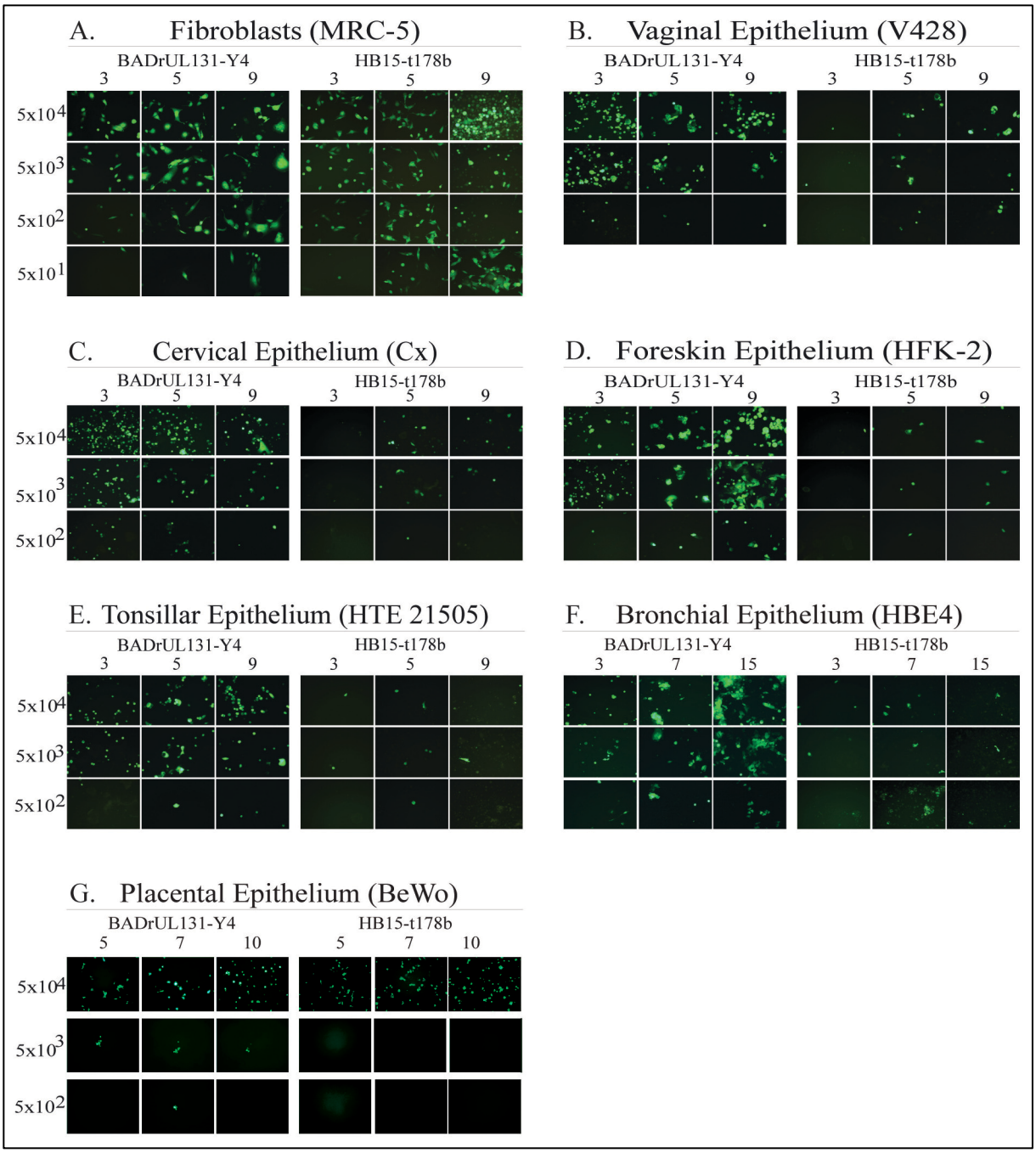
To determine the role of the gH/gL/UL128-131 complex in CMV entry into epithelial cells from mucosal tissues, the entry efficiencies of two GFP-tagged viruses (one expressing and one lacking the gH/gL/UL128-131 complex) were compared by measuring the number of GFP positive cells observed at different times after infection. Strain AD169 is the standard laboratory/reference strain of CMV. It has a frame shift mutation in the *UL131* gene that disrupts expression of the UL131 protein[29] and prevents formation and virion incorporation of the gH/gL/UL128-131 complex.[25] The two viruses used here, HB15-t178b and BAD*r*UL131-Y4, are both AD169-derived, but while HB15-t178b retains the *UL131* mutation and hence fails to express a virion-associated gH/gL/UL128-131 complex, repair of the *UL131* gene in BAD*r*UL131-Y4 restores UL131 expression and virion-incorporation of the gH/gL/UL128-131 complex.[25]

As shown in Fig 15 panel A, the two viral inocula were well matched for entry into MRC-5 fibroblasts even as the inocula were serially diluted down to low levels. Cells originating from genital mucosal tissues, including vagina, cervix, and foreskin, all displayed a pronounced requirement for gH/gL/UL128-131, as evidenced by high levels of GFP positive cells on day 3 following BAD*r*UL131-Y4 infection and a virtual absence of GFP positive cells from cultures that received matching inocula of HB15-t178b (Fig 15, panels B-D). Similar data were obtained

with airway epithelial cells from tonsil and bronchus (Fig 15, panels E and F). Foreskin and bronchial epithelial cells appeared to support the full replication cycle of BAD*r*UL131-Y4, resulting in viral spread, as suggested by increased GFP expression in BAD*r*UL131-Y4-infected cell cultures over time (Fig 15, panels D and F). In contrast, the number of GFP positive cells remained stable over time in BAD*r*UL131-Y4-infected vaginal, cervical, tonsillar, and placental epithelial cells (Fig 15, panels B, C, and E), suggesting a possible post-entry block to BAD*r*UL131-Y4 replication in these cells.

Tumor derived placental epithelial cells did not display a requirement for the gH/gL/UL128-131 complex as indicated by equivalent GFP positive cells at day 5 following infection with HB15-t178b compared to BAD*r*UL131-Y4, and the amount of GFP positive cells remained equivalent between the two viruses at day 10 (Fig 15, panel G). The placental cells were less susceptible to infection with both viruses compared to the other epithelial cell lines (Fig 15, panels B-F) or fibroblast cells. Replication of both viruses was not efficient in the placental cells since the amount of GFP positive cells increased minimally over time and large foci of GFP positive cells, indicating spread, did not develop (Fig 15, panel G).

Figure 15: The gH/gL/UL128-UL131 complex is required for entry into mucosal epithelial cells. Matching inocula of HB15-t178b and BAD*r*UL131-Y4 were 10-fold serial diluted and added to wells of 24-well plates containing confluent cultures of the indicated cells. Cultures were monitored by fluorescence microscopy and photographed on the days indicated after infection. Numbers on the left indicate infectious viral dose (pfu/well).


A. Fibroblasts (MRC-5)
BADrUL131-Y4
HB15-t178b
3 5 9
3 5 9
5x10^4
5x10^3
5x10^2
5x10^1
B. Vaginal Epithelium (V428)
BADrUL131-Y4
HB15-t178b
3 5 9
3 5 9
C. Cervical Epithelium (Cx)
BADrUL131-Y4
HB15-t178b
3 5 9
3 5 9
5x10^4
5x10^3
5x10^2
D. Foreskin Epithelium (HFK-2)
BADrUL131-Y4
HB15-t178b
3 5 9
3 5 9
E. Tonsillar Epithelium (HTE 21505)
BADrUL131-Y4
HB15-t178b
3 5 9
3 5 9
5x10^4
5x10^3
5x10^2
F. Bronchial Epithelium (HBE4)
BADrUL131-Y4
HB15-t178b
3 7 15
3 7 15
G. Placental Epithelium (BeWo)
BADrUL131-Y4
HB15-t178b
5 7 10
5 7 10
5x10^4
5x10^3
5x10^2

Peptides to UL130 and UL131 induce neutralizing antibodies in rabbits

We determined if rabbit sera raised against peptides from UL128, UL130, or UL131 neutralized epithelial cell entry. The rabbit sera were evaluated using a GFP-based neutralizing assay similar to one developed to study sera from naturally infected or experimentally vaccinated humans.[62] Negative control sera from two CMV seronegative donors had no effect on epithelial entry (Fig 16). Positive control sera from six naturally infected donors (see sample information in the appendix) blocked epithelial entry out to dilutions of 1:640 (Fig 16) providing a reference neutralization range for comparison of the rabbit anti-peptide sera activity. Sera obtained from all three rabbits prior to immunization as well as antiserum to the UL128 peptide failed to neutralize epithelial cell entry at any concentration (Fig 16). Rabbit antisera to UL130 or UL131 peptides neutralized epithelial entry with activities within the range defined by the seropositive sera; however, a 50:50 mixture of the anti-UL130 and anti-UL131 sera retained neutralizing activity when diluted four-fold higher than the strongest seropositive human serum (Fig 16).

GFP fluorescence was used to calculate neutralizing titers, assessed as IC_{50} values, for each serum or serum combination. Titers for the six seropositive sera ranged from 1:1007 to 1:3118 with an average of 1:2070. Titers for the antiserum to UL130 (1:6732) or UL131 (1:4096) were slightly above the range defined by the seropositive sera, while that of the UL130+UL131 combination (1:15421) was considerably higher (Fig 17). Interestingly, the epithelial cell neutralizing titer of the UL130+UL131 combination was in the range for the sera from the highly immune individuals described in Table 11.

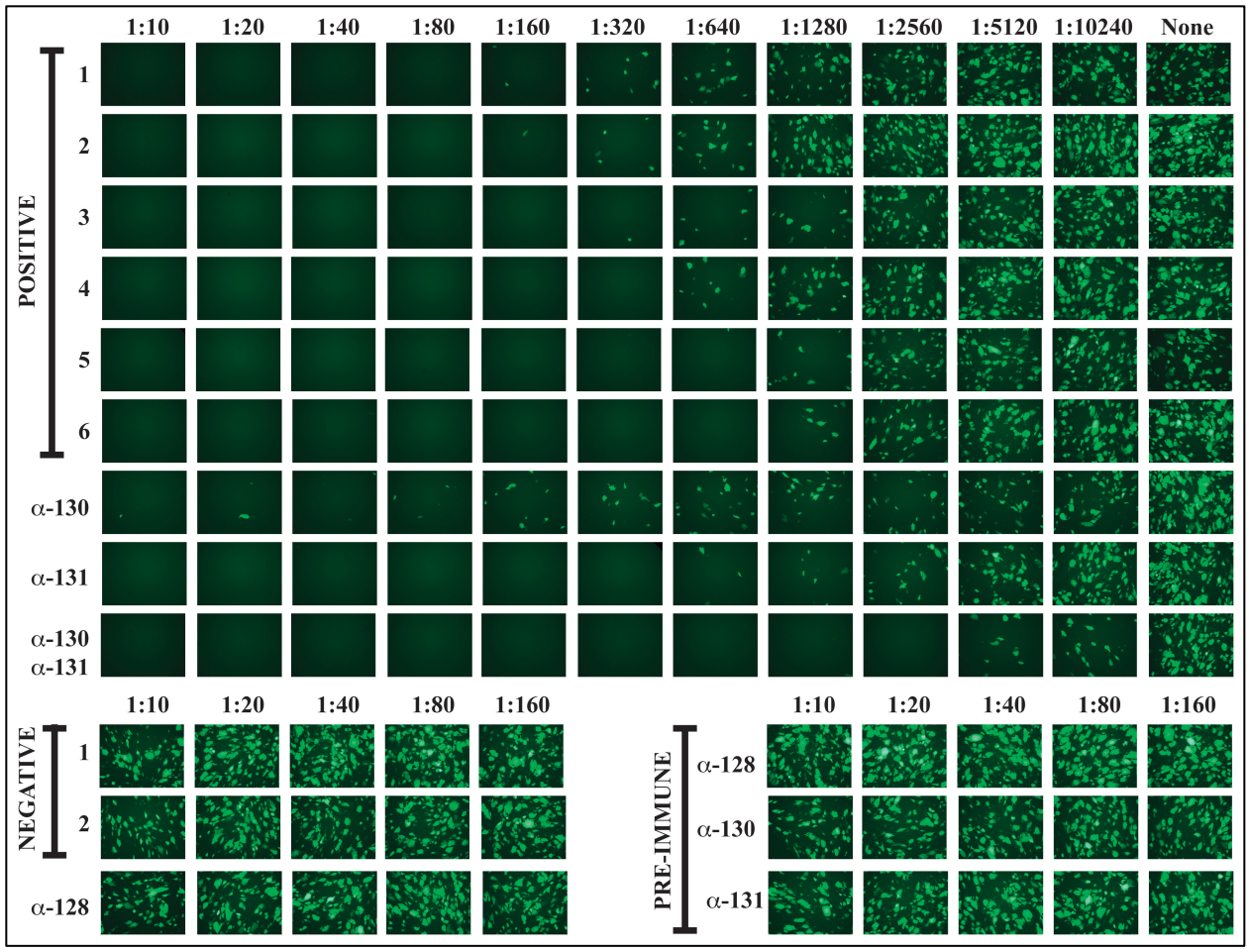


Figure 16: UL130 and UL131 peptides neutralize infection of epithelial cells at least as well as natural infection. The indicated dilutions of sera from six CMV seropositive and two CMV seronegative human subjects, postimmune rabbit anti-peptide sera, and corresponding preimmune rabbit sera were assayed for epithelial entry neutralizing activity. In the bottom row (top panel) equal amounts of rabbit anti-UL130 and anti-UL131 were mixed before being assayed as for the other sera. No serum was added to the wells in the right-most column (top panel). Representative micrographs were taken with a fixed exposure four days post infection.

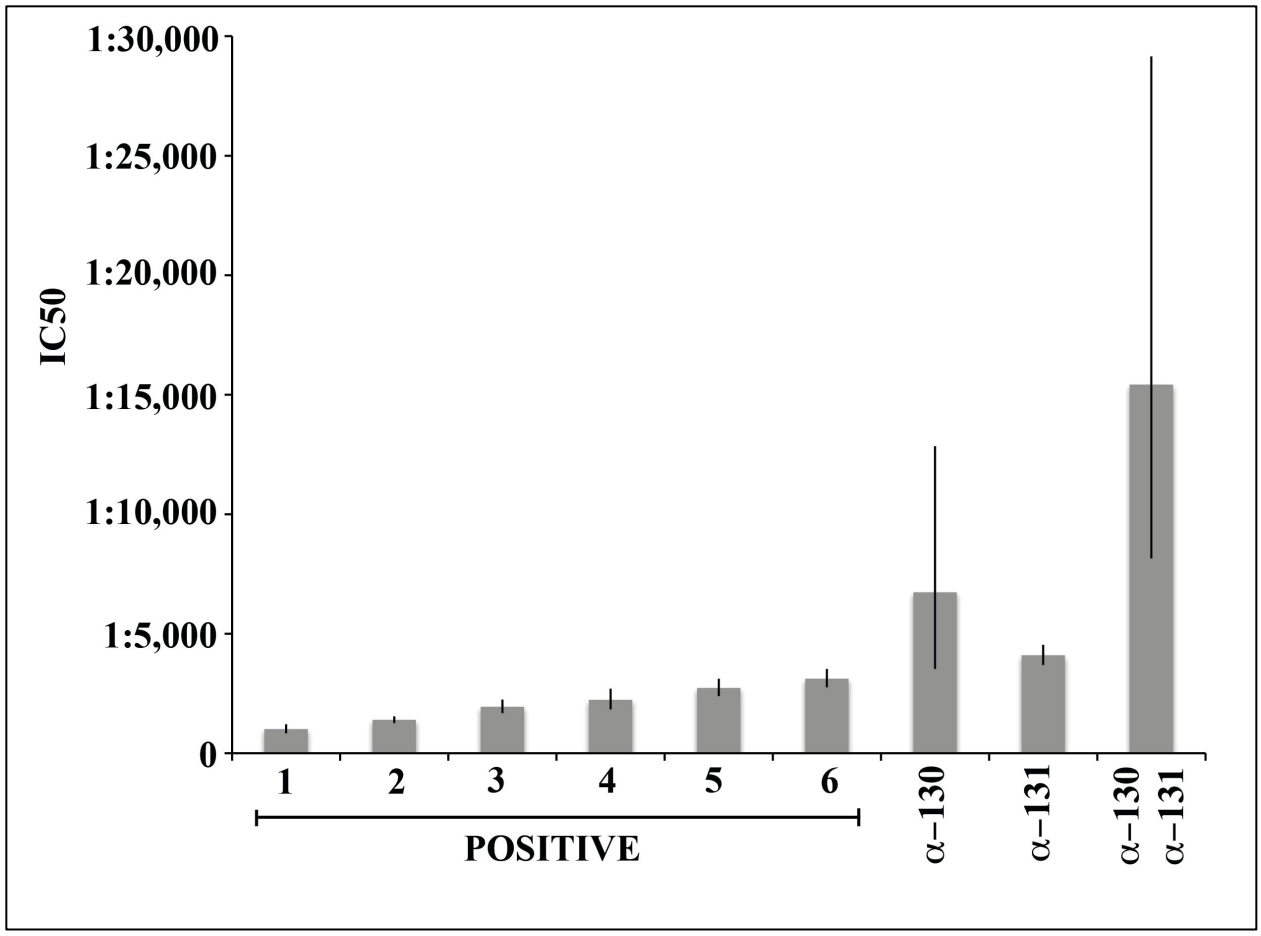


Figure 17: IC_{50} values for human and anti-peptide rabbit sera. IC_{50} values for the same six seropositive human sera shown in Fig 16, the postimmune rabbit anti-UL130 and -UL131 sera, and the mixture of anti-UL130/UL131 sera were calculated using GFP fluorescence values measured from triplicate assays seven days post infection. Error bars indicate standard errors of the means.

Anti-peptide rabbit sera neutralize CMV infection of mucosal epithelial cells

To directly confirm that proteins comprising the gH/gL/UL128-131 complex must be physically present on the virion surface to facilitate viral entry into mucosal epithelial cells, the ability of rabbit anti-peptide sera to block viral entry into cervical, bronchial, and foreskin epithelial cells was determined. As before, the three antisera had no effect on BAD*r*UL131-Y4 entry into fibroblasts (Fig 18). The anti-UL130 and anti-UL131 sera potently inhibited entry to ARPE-19 epithelial cells while the anti-UL128 serum did not (Fig 18). That entry into epithelial cells from cervix, foreskin, and bronchus was highly sensitive to neutralization by both the anti-UL130 and the anti-UL131 sera (Fig 18) physically confirmed that entry into these cell types involves UL130 as well as UL131.

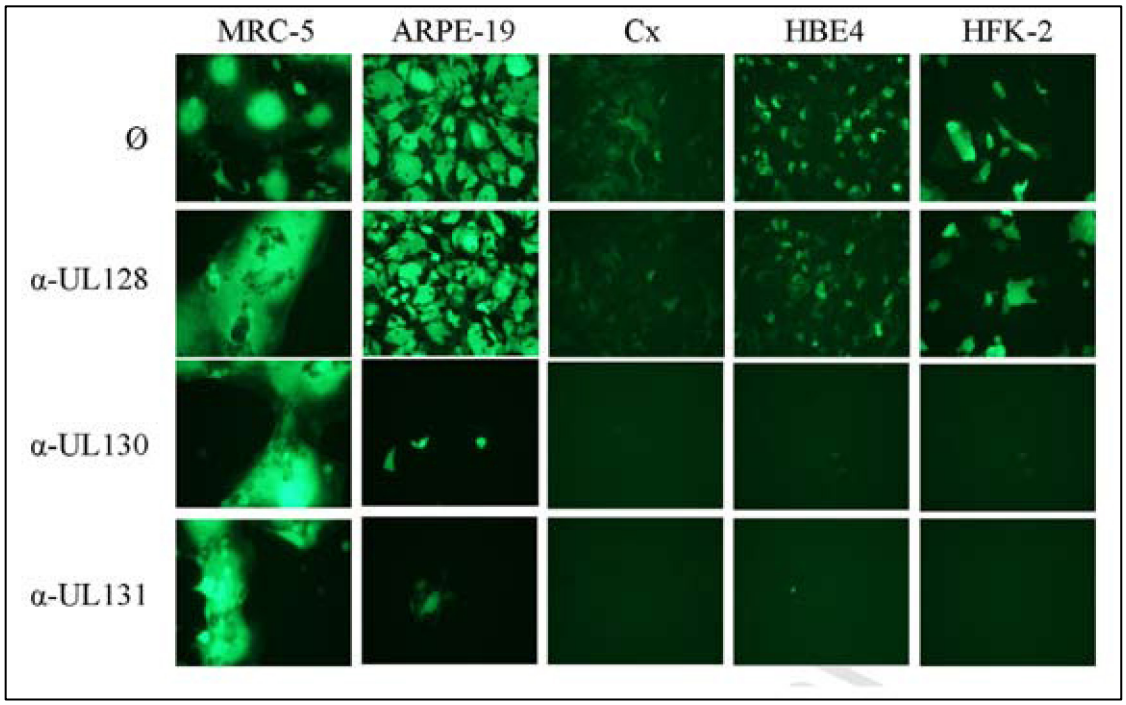


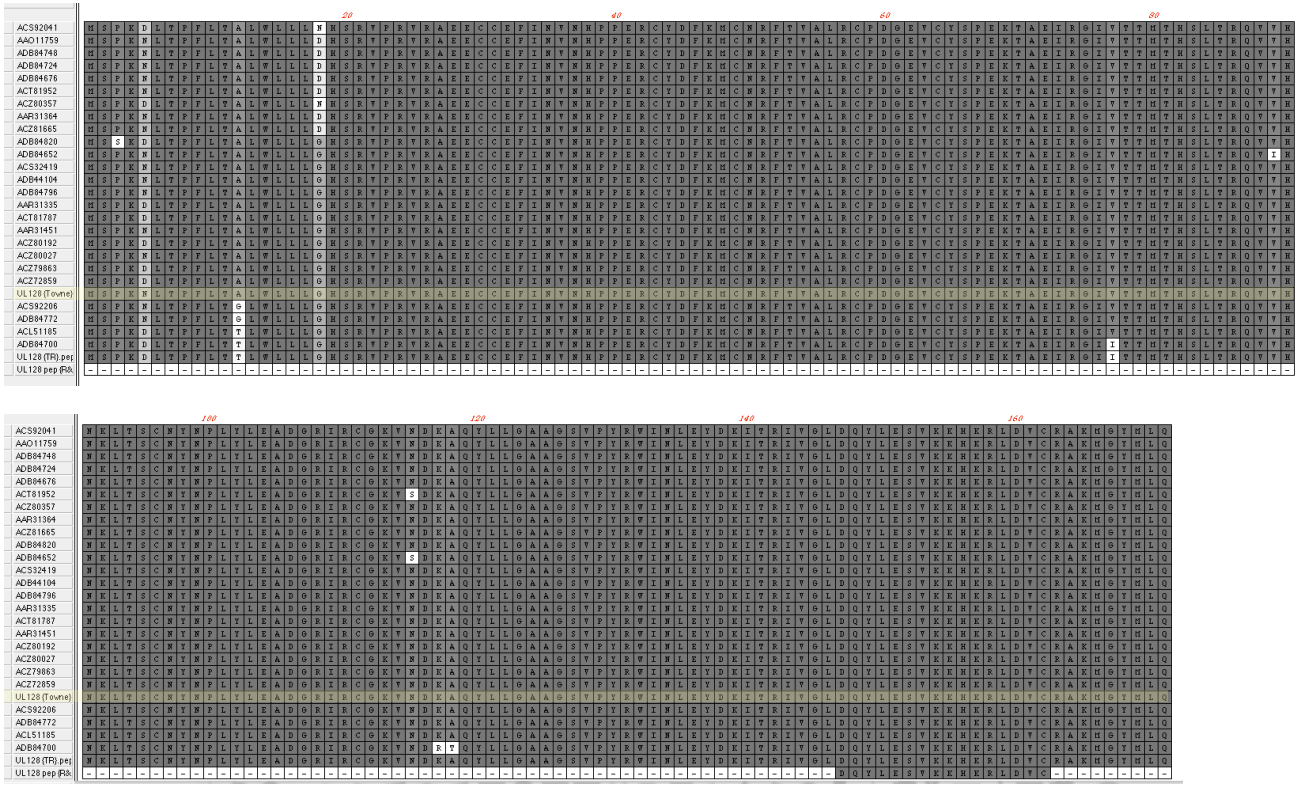
Figure 18: Antibodies to UL130 or UL131 peptides neutralize CMV infection of mucosal epithelial cells. Replicate amounts of BAD*r*UL131-Y4 were mixed with no serum (ø) or 1:20 dilutions of the indicated rabbit anti-peptide antisera. After one hour incubation the mixtures were added to confluent cultures containing the indicated cells and the cultures were monitored daily by fluorescence microscopy. Photographs shown were taken seven days post infection.

Peptides to UL128-UL131 are highly conserved

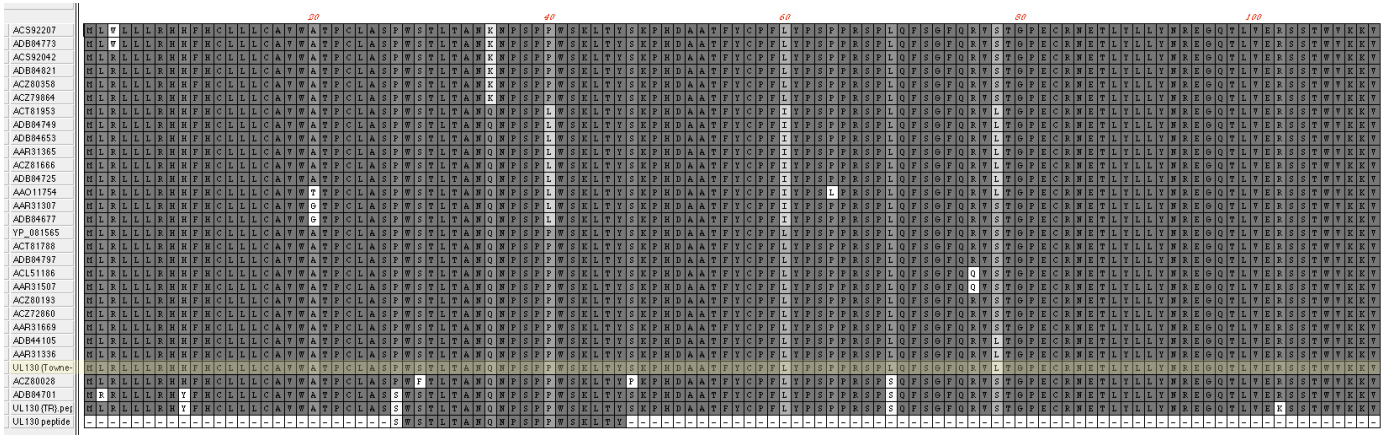
The importance of UL128-131 proteins in epithelial entry makes these proteins attractive vaccine targets. Antigenic variation is important for any potential vaccine immunogen. The UL128-131 proteins are known to be highly conserved between CMV strains,[111] but to specifically determine amino acid variability within the UL128, UL130, and UL131 peptides, DNA sequences from 29 distinct strains available from GenBank were translated and aligned using ClustalW (Fig 19). Nine amino acid positions in UL128 and three in UL131 were polymorphic, but within the UL128 and UL131 peptide regions the amino acid sequences were 100% identical. UL130 was more variable with 19 polymorphic positions resulting in five variants within the UL130 peptide region, as shown in Table 12. These results suggest that antibodies to the UL131 peptide should cross neutralize the majority of CMV strains, whereas antisera raised against the UL130 peptide might be less effective at neutralizing strains expressing different UL130 variants.

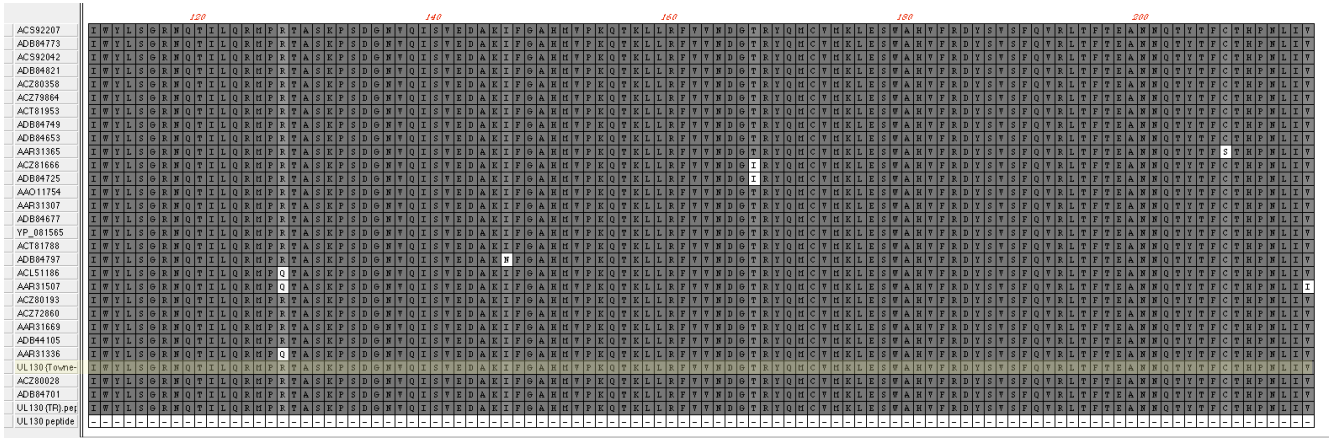
Figure 19: UL128, UL130, and UL131 sequence polymorphisms. CLUSTALW alignments of amino acid sequences for UL128, UL130, or UL131 from strains Towne, TR, AD169, and 29 CMV clinical isolates available from GenBank.

UL128 Alignments



UL130 Alignments





UL131 Alignments

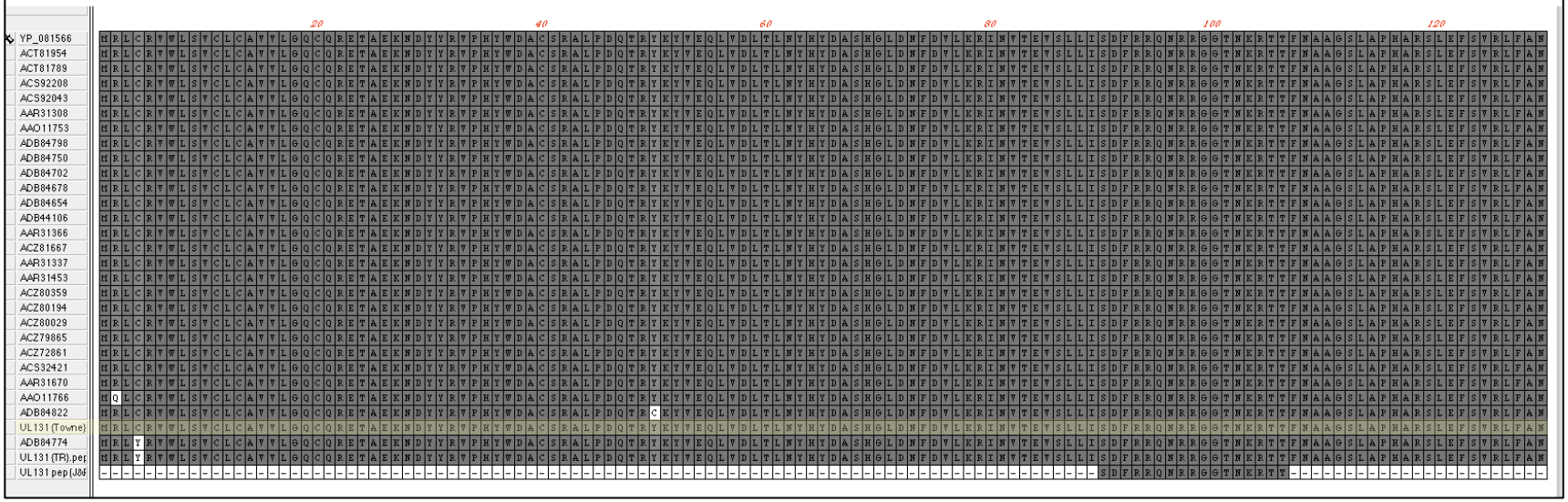


Table 12. Polymorphisms within the UL130 peptide

Variant	*UL130 peptide sequence*[a]	*number of strains*
1	SWSTLTANQNPSPPWSKLTY[b]	2
2	**P**WSTLTANQNPSPPWSKLTY	11
3	**P**W**F**TLTANQNPSPPWSKLTY	1
4	**P**WSTLTAN**K**NPSPPWSKLTY	6
5	**P**WSTLTANQNPSP**L**WSKLTY	9

[a]Amino acid changes relative to the reference strain are shown in bold

[b]Reference strain TR (from which the sequence for the UL130 peptide was derived)

Summary

Neutralizing activity of serum from CMV immune individuals is higher when measured using epithelial cells compared to fibroblast cells.[62] This may be due to the fact that CMV infection of epithelial cells but not fibroblast cells depends on the presence of gH/gL/UL128-131 on the virion surface. Previous data were based on studies using epithelial cells from retinal pigment epithelium and fibroblast cells, but host entry involves CMV infection of mucosal epithelial cells. These observations were expanded to include clinically relevant mucosal epithelial cell lines from the vagina, cervix, foreskin, tonsil, and bronchus. Entry of CMV into these mucosal epithelial cell lines also requires the gH/gL/UL128-131 complex. Similarities between cultured mucosal epithelial cell lines and standard epithelial cell lines (*e.g.* ARPE-19) suggest that data obtained *in vitro* from ARPE-19 cells is translatable to *in vivo* infection.

UL128-131 are potential vaccine targets because of their importance in epithelial cell entry of CMV. Peptides generated to conserved regions of these proteins are immunogenic in rabbits. Anti-peptide rabbit sera to UL130 and UL131 neutralized CMV infection of epithelial cells to similar levels as sera from a panel of six naturally infected individuals. The combination of UL130+UL131 anti-peptide rabbit sera neutralized CMV infection of epithelial cells considerably better than the sera from the six individuals in the panel and reached neutralizing titers similar to that seen in highly immune adults with children in daycare (Neutralizing Activity of Saliva Section). UL130 and UL131 anti-peptide sera also neutralized infection of cervical, foreskin, and bronchial epithelial cell lines. These data support the use of these peptides or the UL128-131 proteins as possible vaccine immunogens in future studies and suggest that epithelial entry neutralizing antibodies in saliva should block CMV entry into mucosal epithelial cells.

DNA VACCINE BASED ON EPITHELIAL ENTRY MEDIATORS UL128, UL130, OR UL131

DNA vaccine development

The first approved veterinary DNA vaccine was for infectious hematopoietic necrosis virus in salmon. A DNA vaccine for West Nile virus in horses is also approved. No DNA vaccines are licensed for use in humans but numerous clinical trials have demonstrated safety and immunogenicity of this vaccine approach. DNA vaccines are attractive vaccine platforms because of their low-cost to manufacture and relative stability without requiring a preservative.[112] DNA injected into skeletal muscle is taken up by both muscle cells and APCs. Muscle cells that express vaccine proteins are able to cross-prime local APCs. DNA vaccines also activate TLR9 to induce innate immunity via plasmid CpG motifs.[113]

DNA vaccines are more effective at inducing T cell responses than B cell responses. Adjuvants are necessary to generate strong antibody responses. Vaxfectin® (Vical, San Diego, CA), a cationic lipid based adjuvant (1:1 mixture of GAP-DMORIE and DPyPE), boosts the immune response to both DNA and protein vaccines. Vaxfectin® complexes with DNA through

charge-based ionic interactions.[114] In animal models Vaxfectin® drastically increases T and B cell responses to various DNA vaccine-encoded antigens by an unknown mechanism.[114-118] Antibody responses in animals vaccinated with Vaxfectin®/DNA vaccine complexes are increased up to 20-fold compared to uncomplexed DNA.[119] An influenza DNA vaccine formulated with Vaxfectin® was safe and immunogenic in a phase I trial.[120]

VCL-CB01 is a gB/pp65 bivalent DNA vaccine that is safe and immunogenic in preliminary clinical trials but preliminary data has not demonstrated protection.[85,86] CMV epithelial entry mediators UL128, UL130, and UL131 are attractive vaccine immonogens because they should generate antibodies that block infection of epithelial cells. UL130 and UL131 anti-peptide rabbit sera neutralize CMV infection of epithelial cells with titers comparable to those of sera from naturally infected adults (Figs 16 and 17). These data support the development of subunit vaccines based on UL128, UL130, and UL131. DNA vaccination with VCL-CB01 plus UL128, UL130, or UL131 may increase the protective immune response induced by vaccination. Alternatively, DNA vaccination with UL128, UL130 and/or UL131 alone may be protective without gB and pp65. DNA vaccination of mice with UL128, UL130, and UL131 will determine the feasibility of these proteins as CMV DNA vaccine immunogens. Since these proteins form a complex and efficient cell surface expression of these proteins requires the expression of UL128, UL130, and UL131,[32] a successful vaccine may require more than one subunit. UL128, UL130, and UL131 DNA vaccines complexed with Vaxfectin® were tested in mice as individual plasmids and in various combinations.

Results

Pilot vaccine study

Expression of pilot DNA vaccine plasmids in vitro

The cDNA sequences encoding *UL128*, *UL130*, and *UL131* (strain TR) were cloned into VR10551 (Vical Inc, San Diego, CA) DNA vaccine plasmid by Xiao Cui as described in the Materials and Methods section to create VR10551-TR128, VR10551-TR130, and VR10551-TR131 (Fig 7). VR10551 contains a kanamycin resistance cassette, CMV IE promoter, CMV intron A from the IE gene, and RBG poly A signal. To verify protein expression, HEK-293T cells were transfected with each plasmid individually, lysed 48 hours post transfection, and used as antigens in western blot assays. Mock-transfected HEK-293T cells were used as a control antigen. Western blots were probed with rabbit anti-peptide sera against each protein. Transfected cells expressed proteins recognized by the appropriate anti-peptide rabbit sera (Figure 20).

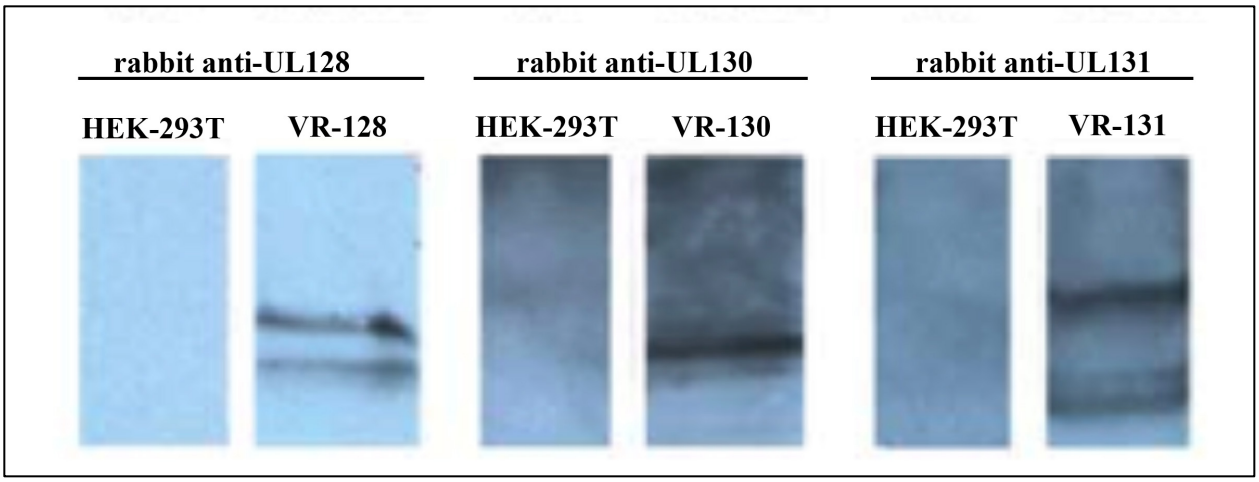
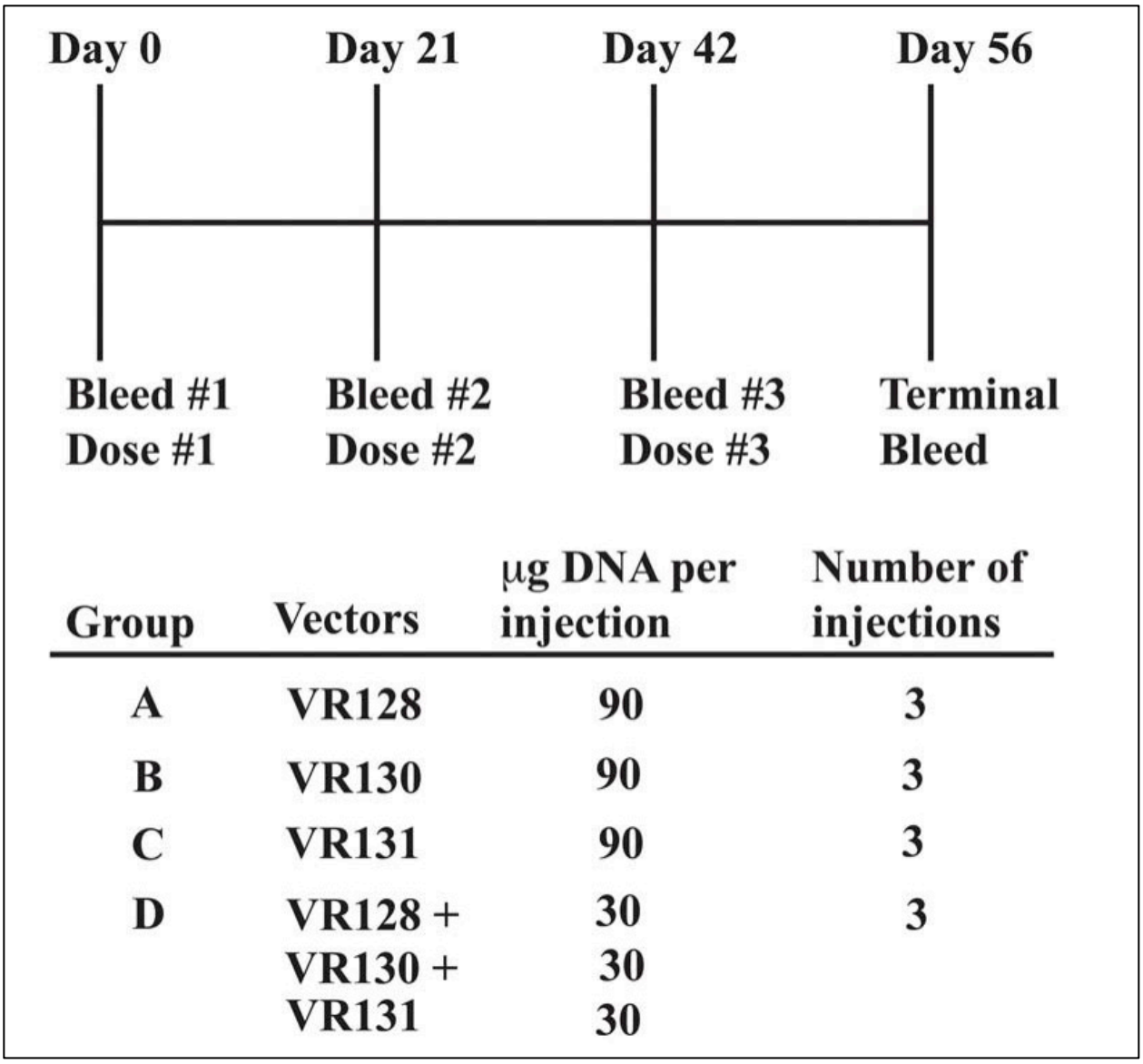


Figure 20: DNA vaccine plasmids express proteins detectable by western blot. HEK-293T cells were mock-transfected (HEK-293T) or transfected with DNA vaccine plasmids VR10551-TR128 (VR-128), VR10551-TR130 (VR-130), or VR10551-TR131 (VR-131) expressing UL128, UL130, or UL131, respectively. Cell lysates were separated by SDS-PAGE, transferred to a nylon membrane, and probed with the indicated rabbit anti-peptide sera to verify recombinant protein expression (X. Cui, unpublished data).

Vaccination schedule

To determine the immunogenicity of the DNA vaccine expression of UL128, UL130, and UL131, Balb/c mice were immunized with plasmids expressing each protein (VR10551-TR128, VR10551-TR130, VR10551-TR131, Fig 7) formulated with Vaxfectin® individually or as a combination of all three plasmids. The vaccination scheme is illustrated in Figure 21. Mice were immunized bilaterally intra-muscularly into the rectus femoris muscle three times every three weeks in four groups of five mice. Sera were collected from each mouse prior to each injection and three weeks after the final dose. Post-immune sera were screened for neutralizing activity on epithelial cells and for antigen-specific antibodies by western blot.



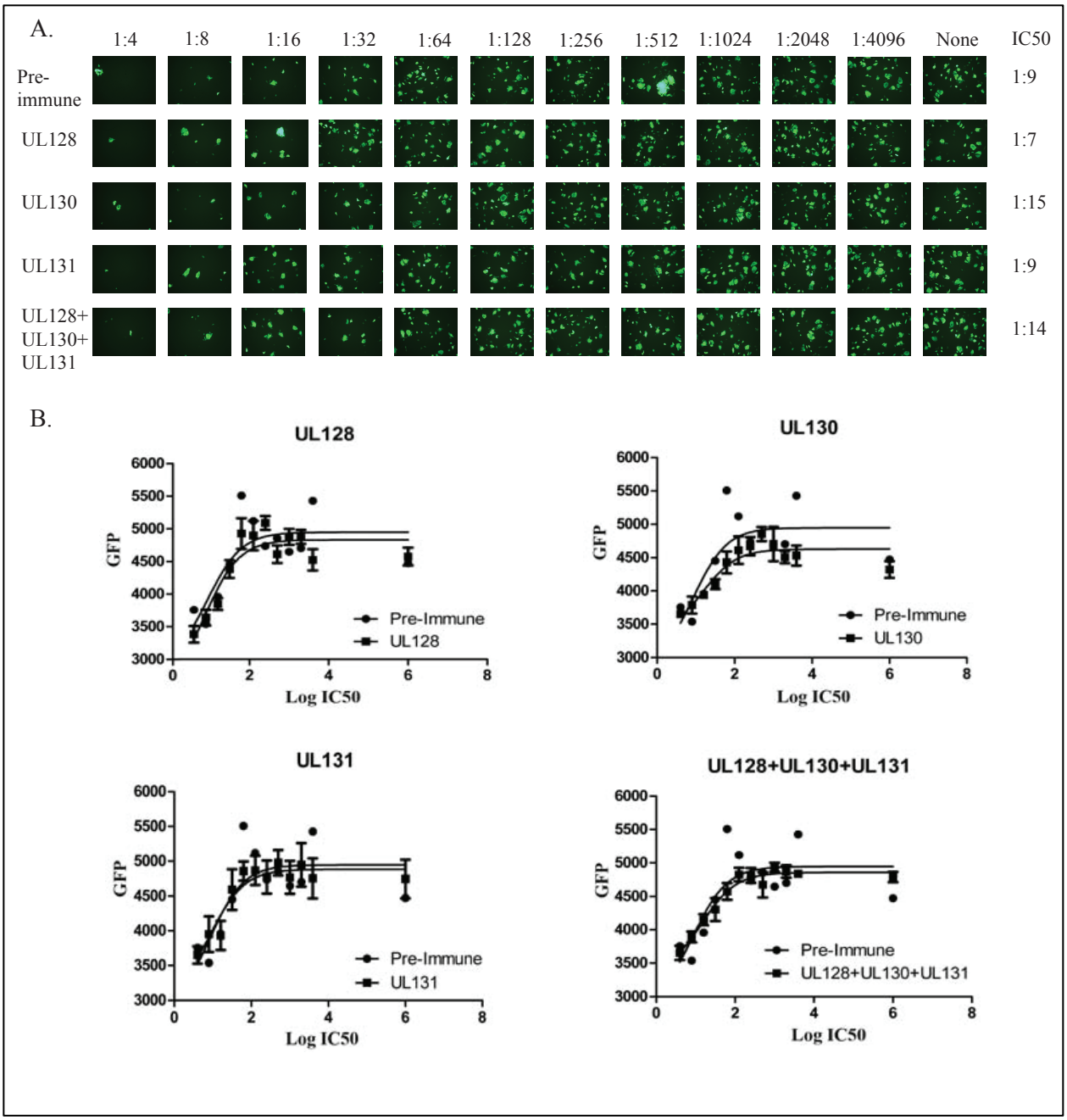
Group	Vectors	μg DNA per injection	Number of injections
A	VR128	90	3
B	VR130	90	3
C	VR131	90	3
D	VR128 + VR130 + VR131	30 + 30 + 30	3

Figure 21: DNA vaccine immunization schedule. Groups of five female Balb/c mice were injected with a total of 90 μg of DNA formulated with Vaxfectin® bilaterally into the rectus femoris muscles three times at three-week intervals.

Vaccinated animals do not develop neutralizing antibodies

Post-immune sera from each mouse were assayed for neutralizing activity on epithelial cells. A single pre-immune serum was used as a control for non-specific mouse serum activity (Fig 22). The pre-immune serum neutralized CMV infection of epithelial cells at a titer of 1:9. Subsequent studies revealed that pre-immune sera from Balb/c mice have non-specific neutralizing activity in this assay that results in background titers of 1:10 to 1:20 (Figures 26-28). Post-immune sera from each mouse were assayed individually and IC_{50} values were calculated for the group based on five replicates (n=5 mice per group). None of the post-immune sera neutralized CMV infection of epithelial cells significantly above the non-specific background titer (see representative data, Fig 22, panel A). The graphs in figure 22, panel B illustrate the mean epithelial cell neutralizing titers of each group and the pre-immune control.

Figure 22: Sera from the initial DNA vaccine study in mice do not neutralize CMV entry. One pre-immune serum and all post-immune sera from each mouse were assayed for epithelial neutralizing activity. Sera were serially diluted two-fold then incubated with 5000 PFU of GFP expressing virus for one hour before being added to epithelial cells. (A) Representative day 5 images from one mouse in each group and one pre-immune control. Average IC_{50} values for each group are shown. (B) Non-linear regression curves based on five replicate sera representing the five mice per group. Each group is plotted with the pre-immune control.


A.
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
1:2048
1:4096
None
IC50
Pre-immune
1:9
UL128
1:7
UL130
1:15
UL131
1:9
UL128+ UL130+ UL131
1:14
B.
UL128
GFP
Log IC50
Pre-Immune
UL128
UL130
Pre-Immune
UL130
UL131
Pre-Immune
UL131
UL128+UL130+UL131
Pre-Immune
UL128+UL130+UL131

Vaccinated animals lack western blot-reactive antibodies to UL128, UL130, and UL131

Since no specific neutralizing activity was detected in post-immune sera, the sera from each mouse were screened for antigen-specific antibodies. HEK-293T cells were infected with adenoviruses expressing UL128, UL130, or UL131 and co-infected with an adenovirus expressing transactivator. Cell lysates for each protein were used as antigens in western blots probed with 10 µl post-immune sera. Anti-peptide rabbit sera were used as controls to confirm expression of each antigen. Post-immune mouse sera did not contain vaccine-specific antibodies detectable by western blot assays (Fig 23). Some mice had antibodies that were reactive with an adenovirus or HEK-293T protein common to all three lysates. One mouse serum in the UL128-vaccinated group recognized a high molecular weight protein (about 70 kDa) much larger than UL128 (15 kDa) in a non-specific manner since this band was present in all three lysates. One UL131 vaccinated mouse serum had antibodies to proteins that appeared to be unique to the Ad5-UL131-infected cell lysate but the molecular weight was much higher (>25 kDa) than UL131 (15 kDa). One mouse in the group vaccinated with UL128+UL130+UL131 recognized a protein about the size of UL130 (25 kDa) but since this protein was present in UL128 and UL131 antigens this is non-specific reactivity to an adenovirus or cellular protein. These data demonstrate that DNA vaccination with UL128, UL130, UL131, or a combination of all three did not induce vaccine-specific antibodies at levels detectable by western blot in any of the mice immunized.

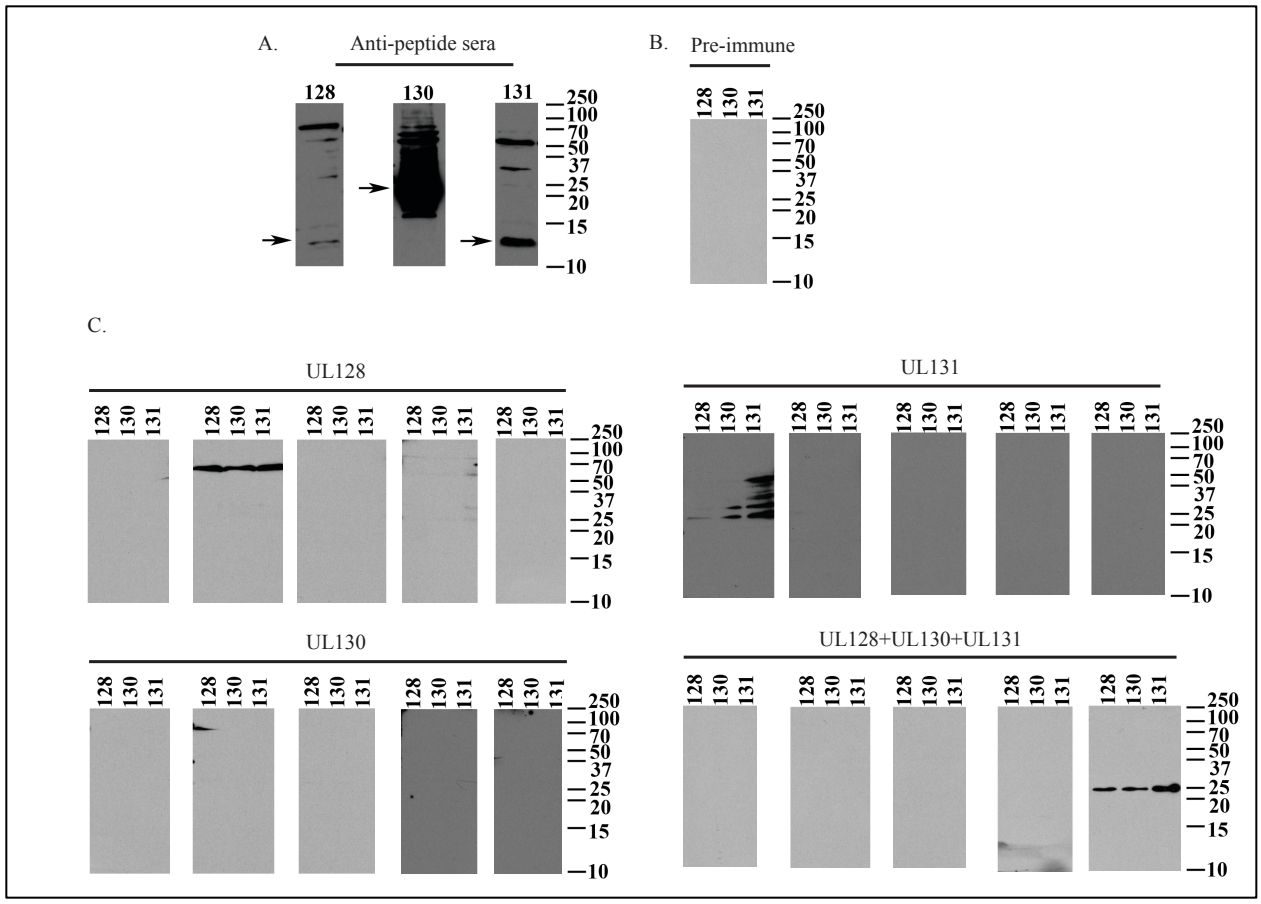


Figure 23: Vaccinated mice lack western blot reactive UL128, UL130, or UL131-specific antibodies. HEK-293T cells infected with Ad5 viruses expressing UL128, UL130, or UL131 were lysed and used as antigens for western blot assays. (A) Blots were probed with the indicated rabbit anti-peptide sera. Arrows indicate the position of UL128 (17 kDa), UL130 (25 kDa), or UL131 (15 kDa). (B) Blot probed with mouse pre-immune serum. (C) Blots probed with post-immune sera from mice vaccinated with the indicated DNA vaccines.

Second generation DNA vaccine study

DNA vaccine redesign, construction, and in vitro expression

Because the first generation vaccine did not induce antibodies detectable by western blot or measurable epithelial neutralization activities, the vaccine plasmids were redesigned to create VR10551-Towne plasmids. First-generation cDNA sequences were cloned directly from strain TR and were not codon optimized. To maximize expression, second-generation DNA vaccines were designed and constructed using codon-optimized sequences cloned into VR10551. Gene sequences from strain Towne were used instead of TR sequences because the Towne sequences were more broadly representative of clinical strains in GenBank (Fig 19).

Prior to immunization, UL128, UL130, and UL131 protein expression of the second-generation DNA vaccine plasmids were confirmed using western blot assays. HEK-293T cells were transfected with each plasmid individually and cell lysates were used as western blots antigens. Anti-peptide rabbit sera were used to verify protein expression. The second-generation DNA vaccine plasmids expressed each protein at levels detectable by the appropriate anti-peptide rabbit sera (Figure 12). Protein expression in cell lysates was comparable between the first and second-generation DNA vaccine plasmids (Figure 24). These data suggest that codon-optimization did not significantly alter gene expression of the plasmid-encoded proteins *in vitro*. However, western blot data are not highly quantitative. Given that the UL130 signal from the second-generation vector extends a little lower, it is possible that there was some improvement in the UL130 expression. It is also important to note that the TR peptide used to raise the rabbit serum differs at the first amino acid from the Towne UL130 sequence (S instead

of P). Although improbable, this difference could make the rabbit serum less efficient at recognizing the Towne UL130 protein vs. TR UL130.

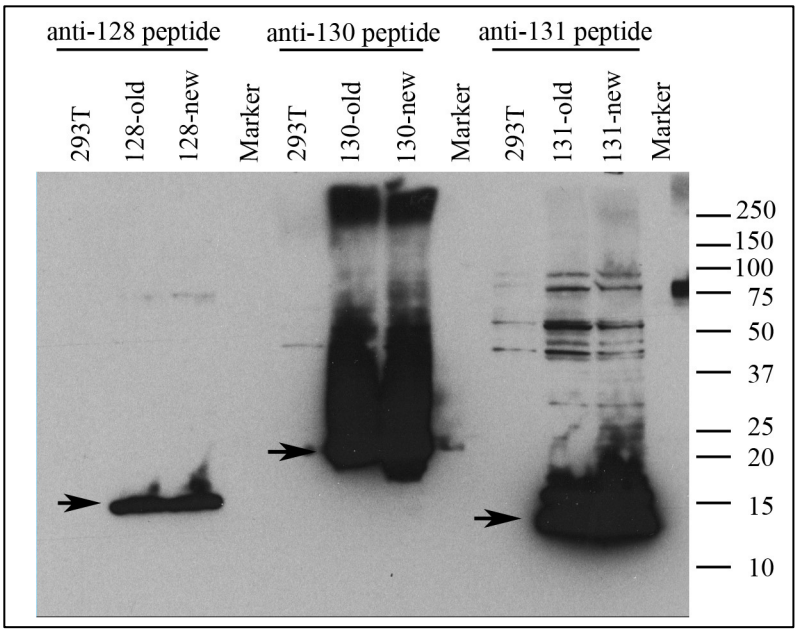


Figure 24: Second-generation DNA vaccine plasmids express UL128, UL130, and UL131 at comparable levels to the first-generation vaccine plasmids. HEK-293T cells were transfected with either second-generation codon-optimized DNA vaccine plasmids (new; VR10551-Towne) or the first-generation DNA vaccine plasmids (old; VR10551-TR) and used as antigens in western blot assays. Mock-transfected cells were used as control antigens (293T). Anti-peptide rabbit sera were used to detect the corresponding proteins. Positions of molecular weight markers are shown on the right. Arrows indicate the positions of UL128 (17 kDa), UL130 (25 kDa), and UL131 (15 kDa).

Vaccination of animals with the second generation DNA vaccine

The second-generation DNA vaccine plasmids formulated with Vaxfectin® were tested in a larger group of mice (n=80) according to the vaccination schedule in Figure 21. Female Balb/c mice were divided into eight groups with ten mice per group. Vaccine groups are listed in Table 13. One mouse in group 5 died of non-vaccine related causes before the terminal bleed. The gB DNA vaccine developed by Vical Inc (San Diego, CA) was used as a control. This gB plasmid is included in Vical's DNA vaccine clinical trials and induces high gB-ELISA titers in mice and humans.[85,105] Mouse vaccination and sera collection was performed by Vical, Inc. at the Explora Vivarium in San Diego, CA. Serum samples were shipped to VCU for analysis.

Table 13: Second-generation DNA vaccine groups

Group	Vectors	μg DNA per injection	Number of injections
1	UL128	100	3
2	UL130	100	3
3	UL131	100	3
4	UL128+UL130	50, 50	3
5	UL128+UL131	50, 50	3
6	UL130+UL131	50, 50	3
7	UL128+UL130+UL131	33, 33, 33	3
8	gB	100	3

gB vaccinated mice lack epithelial cell neutralizing activity

The gB vaccine plasmid used in this study has been used in clinical trials but sera from gB-DNA vaccinated mice have never been tested for neutralizing activity on epithelial or fibroblast cells. The history of successful antibody response following gB immunization of mice, rabbits, and humans supported the use of this plasmid as a positive control.[85,105] Pre and post-immune sera from each vaccinated mouse were initially screened at a 1:30 dilution in the epithelial cell entry neutralizing assay with the hypothesis that sera that neutralize viral entry at 1:30 will give significantly lower GFP values than those that do not. However, that the GFP values were not decreased for gB-DNA vaccinated mice in the 1:30 screen suggested that these mice did not produce epithelial entry neutralization activity (Fig 25). Sera from mice 8-7 and 8-10, which had the lowest GFP values on the 1:30 screen, were assayed for neutralizing activity on both epithelial and fibroblast cells. These sera did not have any neutralizing activity above the pre-immune background on either cell type (Fig 26, panel A). This result was unexpected because this vaccine plasmid induces high gB-specific ELISA titers in mice.[105] Collaborators at Vical tested the gB-vaccinated post-immune sera for gB ELISA activity. All ten sera were highly positive for gB antibodies, indicating that the mice did make gB-specific antibodies but these antibodies do not neutralize CMV in our assay.

To determine if the gB plasmid can induce neutralizing antibodies, pre and post immune sera from four gB-DNA vaccinated rabbits with high gB ELISA titers (a kind gift from Vical, Inc San Diego, CA) were assayed on epithelial cells. These sera had neutralizing activity (average titer 1:25; range 1:7-1:31) on epithelial cells but the titers were less than that of sera

from human subjects that received the gB/MF59 subunit vaccine (average titer 1:64)[62] (Fig 26, panel B).

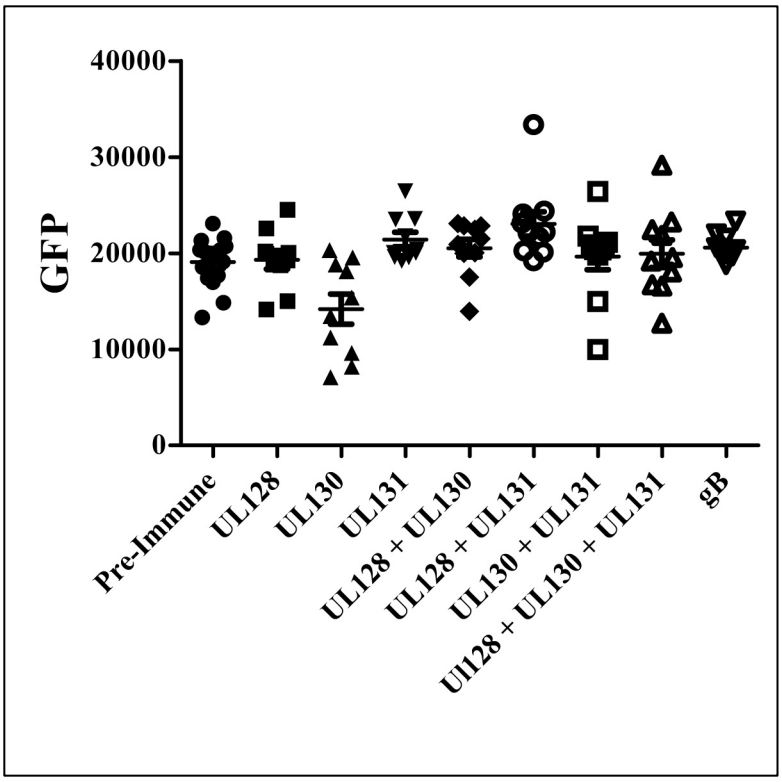


Figure 25: 1:30 screen for epithelial entry neutralizing activity from DNA-vaccinated mice. Post-immune sera from each mouse were screened at a single 1:30 dilution for epithelial entry neutralizing activity, recorded as GFP fluorescence on day seven. Two pre-immune sera from each vaccine group were randomly selected to comprise the pre-immune group shown here.

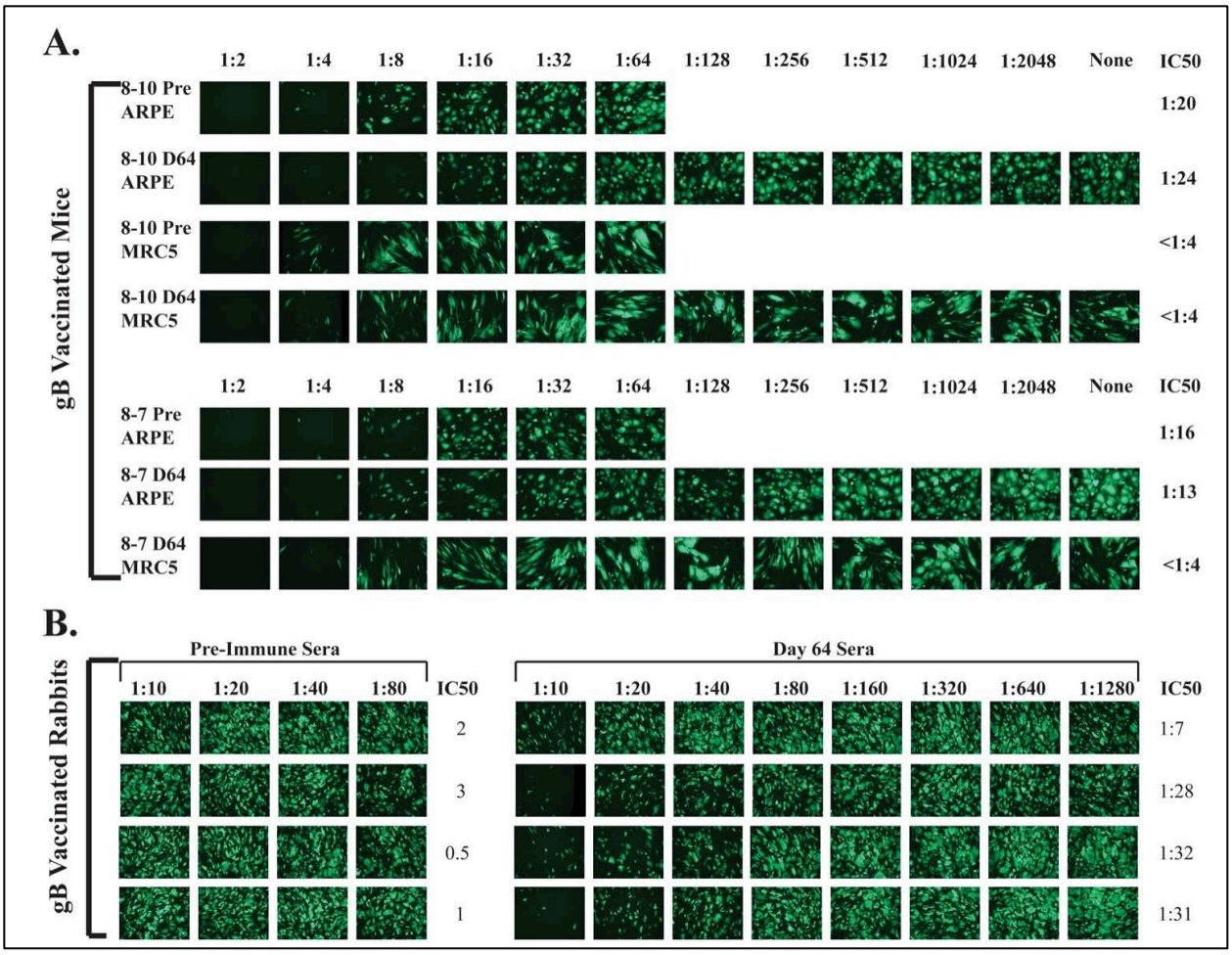
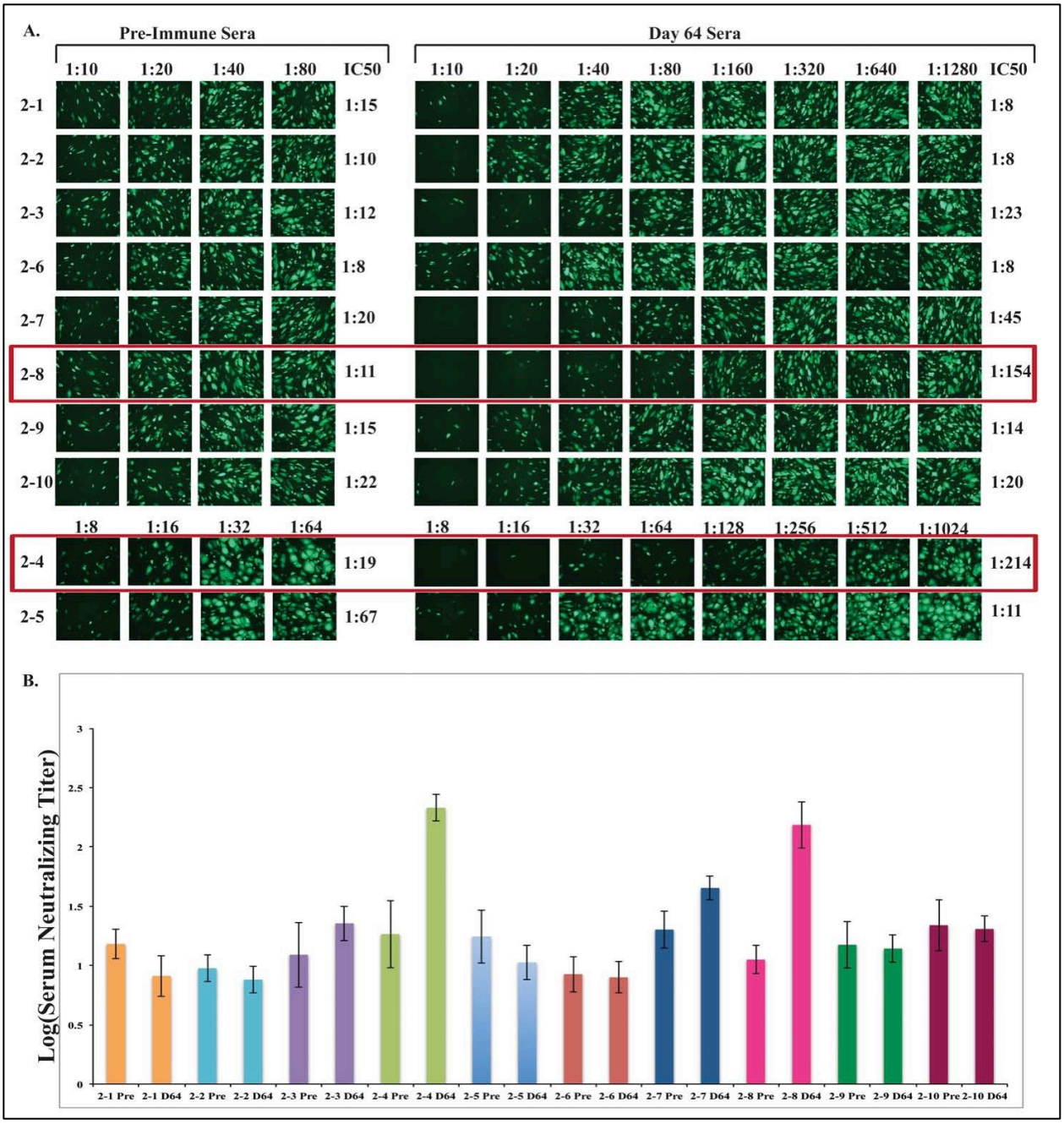


Figure 26: gB-DNA vaccination of rabbits but not mice induces neutralization activity. (A) Pre and post immune (day 64) sera from two gB DNA-vaccinated mice were assayed in triplicate for neutralizing activity on epithelial (ARPE-19) and fibroblast (MRC-5) cells. IC_{50} titers for each serum are shown to the right. (B) Pre and post immune (day 64) sera from four gB-vaccinated rabbits were assayed in triplicate for epithelial cell neutralization activity. IC_{50} values are shown to the right.

Some UL130-Vaccinated animals developed neutralizing antibodies

Mean GFP values from the 1:30 screen for the UL130-vaccinated group were statically lower than other groups. No other vaccine group had mean epithelial GFP values lower than the pre-immune group (Fig 25). Based on the 1:30 screen results all sera from the UL130 vaccine group were assayed for epithelial entry neutralizing titers. Mice 2-4 and 2-8 had titers of 1:214 and 1:154 respectively. Mice 2-3 and 2-7 had epithelial neutralizing titers approximately double background but this activity was within the standard error of the pre-immune control for each mouse and therefore not considered to be above background. No other mice in the UL130-vaccinated group had neutralizing titers above pre-immune background (Fig 27). Several other mice from other groups had low GFP values suggestive of neutralizing activity in the 1:30 screen (one mouse in the UL128+UL131 group; two mice in the UL130+UL131 group; one mouse in the UL128+UL130+UL131 group). Pre and post immune sera from these animals were assayed for epithelial entry neutralizing activity. No activity was found above background for any of these sera (Fig 28).

Fig 27: Two of ten UL130-vaccinated mice have epithelial entry neutralizing activity. Pre and post immune (day 64) sera from the ten mice in the UL130 DNA vaccine group were assayed in triplicate for neutralizing activity on epithelial cells. Two mice, 2-4 and 2-8, had neutralizing activity above background and are highlighted by red boxes. (A) Representative day 4 images and IC_{50} values as determined by non-linear regression of triplicates. (B) Histogram of pre and post immune IC_{50} values (color-matched for each mouse). Error bars represent standard error.


A.
Pre-Immune Sera
Day 64 Sera
1:10
1:20
1:40
1:80
IC50
1:160
1:320
1:640
1:1280
2-1
1:15
1:8
2-2
1:10
1:8
2-3
1:12
1:23
2-6
1:8
1:8
2-7
1:20
1:45
2-8
1:11
1:154
2-9
1:15
1:14
2-10
1:22
1:20
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
2-4
1:19
1:214
2-5
1:67
1:11
B.
Log(Serum Neutralizing Titer)
0
0.5
1
1.5
2
2.5
3
2-1 Pre
2-1 D64
2-2 Pre
2-2 D64
2-3 Pre
2-3 D64
2-4 Pre
2-4 D64
2-5 Pre
2-5 D64
2-6 Pre
2-6 D64
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2-8 Pre
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2-9 Pre
2-9 D64
2-10 Pre
2-10 D64

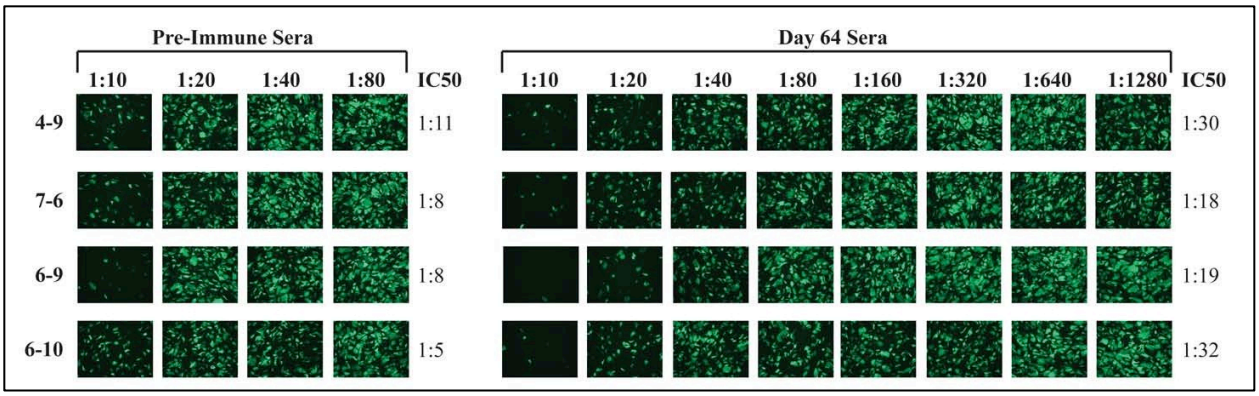


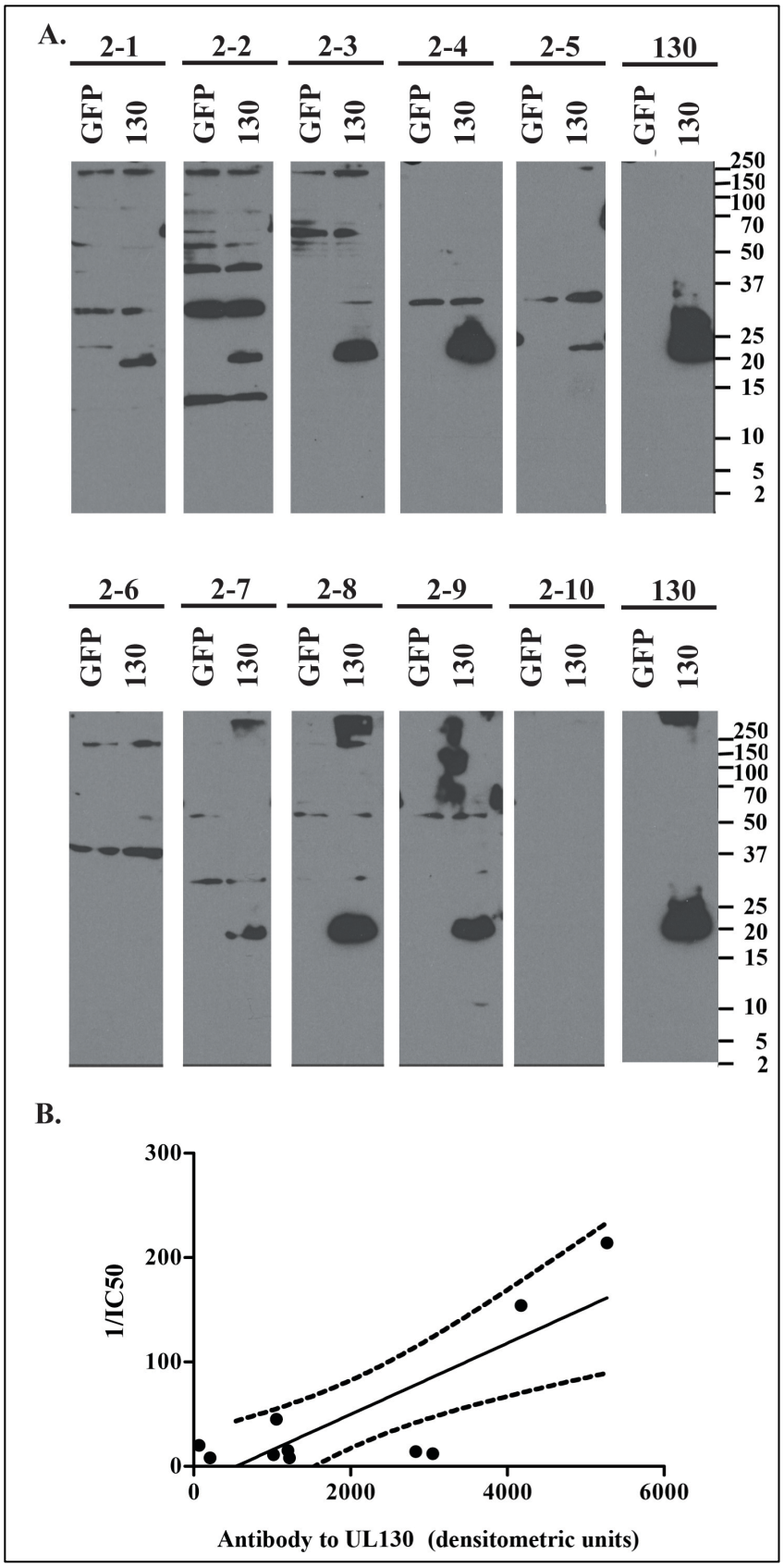
Figure 28: Other sera identified in the 1:30 screen lack significant neutralizing activity.

Pre and post immune sera from mouse 4-9 (UL128+UL130), mouse 6-9 (UL130+UL131), mouse 6-10 (UL130+UL131), and mouse 7-6 (UL128+UL130+UL131) were assayed in triplicate for neutralizing activity on epithelial cells. Representative day 4 images and IC_{50} values (shown to the right of the images) determined by non-linear regression of triplicates are shown.

Most UL130-vaccinated mice have antibodies to UL130

Lack of detectable epithelial entry neutralizing activity in the majority of immunized mice may be due to a lack of induction of vaccine-specific antibodies. Since 2 of 10 mice in the UL130 group had detectable neutralizing activities on epithelial cells, post-immune sera from the UL130 group were screened for the presence of UL130 antibodies by western blot. Lysates from HEK-293T cells transfected with VR10551-Towne130 or cells transfected with a plasmid expressing a control protein (GFP) were used as western blot antigens. Blots were probed with 10 µl of UL130-vaccinated mouse sera. Eight of ten sera contained detectable UL130 antibodies. Mice 2-4 and 2-8, which had detectable epithelial neutralizing activity, had the strongest UL130 western blot signals. Six mice that did not have epithelial neutralizing activity also had UL130 signals (Fig 29, panel A). Densitometric quantitation of the UL130 western blot signals showed a positive correlation with epithelial entry neutralizing titers (Fig 27, panel B; linear regression R^2= 0.6657, r= 0.8159, p= 0.0040), suggesting that insufficient antibody production was the primary reason that most mice in this group lacked epithelial entry neutralizing activity.

Figure 29: Most UL130-vaccinated mice have serum antibodies to UL130. (A) HEK-293T cells were transfected with VR10551-Towne130 or a GFP control plasmid, lysed at 48 hours post transfection, and used as antigens for western blot assays. Blots were probed with post immune sera from the indicated mice or rabbit anti-peptide UL130 serum (used as a positive control). Arrows indicate the location of UL130 (25 kDa). (B) Densitometric quantitations of UL130 western blot signals were determined by ImageJ and plotted vs. reciprocal epithelial entry neutralizing titer (IC_{50}). Linear regression was used to determine the correlation between IC_{50} titer and densitometry. Solid line indicates best-fit line determined by linear regression. Dashed lines indicate 95% confidence intervals.


A.
2-1
2-2
2-3
2-4
2-5
130
GFP
130
250
150
100
70
50
37
25
20
15
10
5
2
2-6
2-7
2-8
2-9
2-10
130
B.
300
200
100
0
1/IC50
0
2000
4000
6000
Antibody to UL130 (densitometric units)

Most UL128 and UL131 vaccinated mice did not have vaccine-specific antibodies

Since 80% of UL130-vaccinated mice have vaccine-specific antibodies by western blot, day 64 post-immune sera from vaccinated mice in other groups were tested by western blot for vaccine specific antibodies. Lysates from HEK-293T cells transfected with VR10551-Towne128, VR10551-Towne130, or VR10551-Towne131 were used as western blot antigens. Blots were probed with 10 µl of day 64 vaccinated mouse sera from the UL128, UL130, UL131, UL128+UL130, UL130+UL131 and UL128+UL130+UL131 groups (Table 14). One of the UL128-vaccinated mice had detectable UL128-reactive antibodies by western blot. Data in Figure 29 were confirmed with eight of the UL130-vaccinated mice having detectable UL130-specific antibodies. None of the UL131-vaccinated mice had detectable UL131 antibodies. One of the UL128+UL130 vaccinated mice had detectable UL130-reactive antibodies by western blot. Six of the UL130+UL131 vaccinated mice had UL130-specific antibodies, one of the vaccinated mice had UL131 antibodies, and none of the UL130+UL131 vaccinated mice antibodies to both proteins. The UL128+UL130+UL131 vaccinate group had six mice with UL130-reactive antibodies by western blot (Table 14).

Table 14: DNA vaccine-specific antibodies detectable by western blot

Vaccine	N	Western blot reactivity	Neutralization
UL128	10	1	0
UL130	10	8	2 (1:154; 1:214)
UL131	10	0	0
UL128+UL130	10	1	0
UL128+UL131	9	n.d. [a]	0
UL130+UL131	10	6 (UL130); 1 (UL131)	0
UL128+UL130+UL131	10	6 (UL130)	0

[a] not determined

Summary

Sera from the majority of vaccinated mice in both studies failed to neutralize CMV infection of epithelial cells. Post-immune sera from mice in the first study immunized with UL128, UL130, UL131, or UL128+UL130+UL131 did not contain epithelial entry neutralizing activity or vaccine-specific antibodies detectable by western blot. Codon-optimization did not significantly increase the *in vitro* expression of UL128, UL130, or UL131 but it may have increased the immunogenicity of the vaccine *in vivo* since 8/10 mice vaccinated with UL130, 6/10 UL130+UL131, and 6/10 UL128+UL130+UL131 vaccinated mice made UL130-specific antibodies detectable by western blot. Two of the UL130-vaccinated mice had epithelial entry neutralizing activities of 1:154 and 1:214 (Figure 27). The increase in immunogenicity may also be due to vaccination performed by more experienced personnel. Interestingly, DNA vaccination of mice with gB did not induce neutralizing antibodies.

Minimal immunogenicity of this DNA vaccine may be due to the instability of these proteins when expressed individually.[32] Co-immunization with multiple plasmids did not increase immunogenicity indicating that there is no advantage in vaccinating with all three plasmids or pairs to form potential multi-subunit conformational epitopes. This may be due to inefficient uptake and/or expression of multiple plasmids by the same cell. The presence of UL130 antibodies in 8/10 mice vaccinated with UL130, 6/10 UL130+UL131, and 6/10 UL128+UL130+UL131 vaccinated mice, and neutralizing antibodies in 2/10 mice vaccinated with UL130 suggests that UL130 may be the best DNA vaccine candidate for a monovalent subunit vaccine, but consistency is an issue.

DISCUSSION

Deficiencies of existing vaccines

Congenital CMV is the leading cause of birth defects in the U.S., affecting about 1% of neonates born each year. A large number of these infected children will develop sensorineural hearing loss.[3] Due to the high disease impact, development of a CMV vaccine was deemed a priority by the Institute of Medicine.[60] Over the past 40 years CMV vaccine development has focused on inducing neutralizing titers on fibroblast cells, specifically to gB, but recent data suggest that epithelial entry neutralizing antibodies may be more protective.[62] Therefore, the focus of subunit vaccine development on gB may be misguided.

The gB/pp65 DNA vaccine reduces time to CMV reactivation, time on anti-virals, and severity of disease in transplant patients but this vaccine does not induce high antibody responses.[105] This could be a result of the DNA vaccine approach, which induces stronger T cell than antibody responses. It is possible that relying primarily on gB for a protective antibody response is the flaw of this vaccine. Immunizing with gB and pp65 is unlikely to induce high epithelial entry neutralizing activity since these proteins do not provide antigenic targets specific for the epithelial entry pathways, such as UL128, UL130, and UL131. Antibodies from

seropositive individuals to epithelial entry mediators UL128, UL130, and UL131 have potent neutralizing activities.[52] gB-specific antibodies constitute the majority of anti-CMV antibodies but they may not be as potent as UL128, UL130, and UL131 antibodies, which appear to be present at much lower concentrations.

The gB/MF59 protein subunit vaccine induces serum gB titers approximately five-fold above natural infection, but is only 50% protective in women of child bearing age and solid organ transplant recipients.[61,65,121] These data suggest that high titer antibodies to moderately potent epitopes in gB are not completely protective and that inducing even higher gB titers may not compensate for the absence of more potent antibodies to epitopes in epithelial entry mediators. Sera from gB/MF59 vaccine recipients have fibroblast entry neutralizing activities similar to natural infection (natural infection mean 1:20; gB MF59 mean 1:16; $p = 0.113$) but compared to natural infection have much lower epithelial entry neutralizing activity (natural infection mean 1:944; gB MF59 mean 1:65; $p < 0.0001$).[62] This vaccine induces some mucosal immunity since 50% of vaccinated subjects have gB-specific sIgA in their saliva[51] but it is not known if these salivary antibodies are sufficient to neutralize virus. It is possible that gB-specific sIgA or IgG levels in saliva correlate with protection but unfortunately this correlation cannot be determined from current published data.

The Towne vaccine, which has a *UL130* gene mutation that prevents expression of gH/gL/UL128-131 on the virion surface,[33] induces minimal mucosal immunity and no salivary epithelial entry neutralizing activity. Salivas from Towne vaccine recipients contain some gB specific IgG, but no sIgA or IgA as measured by ELISA.[51] The lack of mucosal immunity following Towne vaccination most likely results from the low levels of serum humoral immunity

induced by the Towne vaccine compared to natural infection.[122] Sera from Towne vaccine recipients does not replicate the high level of epithelial entry neutralizing activity seen following natural infection (Towne mean 1:34; natural infection mean 1:944; $p < 0.0001$) but fibroblast entry neutralizing activities are similar (1:16 for Towne; 1:20 for natural infection; $p = 0.0418$).

It is not known if Towne vaccinees develop antibodies to UL128, UL130, or UL131. The *UL130* gene mutation in Towne causes rapid degradation of the protein[33] so vaccine recipients presumably do not generate an immune response to UL130 or epitopes that rely on the assembled gH/gL/UL128-131 complex. Antibodies to UL128 and UL131 may still be generated following Towne vaccination, but conformation-dependent antibodies to the gH/gL/UL128-131 complex will be lacking. Interestingly, a subset of mice vaccinated with a DNA vaccine for UL130 but not UL128 or UL131 generate vaccine-specific neutralizing antibodies (DNA Vaccine Section). This suggests that UL130 is more immunogenic than UL128 or UL131 as a single immunogen. Thus, for a live attentuated vaccine, the *UL130* mutation in Towne may impact neutralizing titers more so than would mutations affecting *UL128* or *UL131* (and not *UL130*) since vaccination with UL130 alone can induce measurable neutralizing antibodies. Indeed, immunization of seronegative subjects with the four Towne/Toledo chimeras (now underway) should provide serum samples to test this hypothesis, as all four of these candidate strains are predicted to be wild type for *UL130* and mutant for *UL128*. An ideal vaccine, however, would express an antigenically native gH/gL/UL128-131 complex.

Importance of mucosal immunity in vaccine development

Mucosal immunity, particularly salivary antibodies, will be a necessary part of a successful CMV vaccine. Serum antibodies produced following natural infection neutralize

CMV entry into epithelial cells at much higher titers than fibroblast cells.[62,63] Current vaccines focus on inducing gB-specific antibodies, which will theoretically target both fibroblast and epithelial cell entry since gB is required for entry into both cell types. However, potency of gB epitopes appears low compared to epithelial entry epitopes in gH/gL/UL128-131.

Vaccination with epithelial entry mediator targets presumes, however, that infection of mucosal epithelial cells is gH/gL/UL128-131-mediated and hence neutralizable with gH/gL/UL128-131-specific antibodies. To date, the majority of published work on the mechanism and neutralizing activities against epithelial entry have used ARPE-19 cells, which are derived from the retinal pigment epithelium of the eye. The importance of gH/gL/UL128-131 for viral entry has also been confirmed for tumor cells of epithelial origin derived from breast, cervix, lung, and colon.[25]

Here, CMV entry into cells derived from tissues believed to be most relevant to CMV acquisition, airway and genital mucosa was evaluated, and in all cases found that entry is gH/gL/UL128-131-dependent. Interestingly, placental epithelial cells did not require gH/gL/UL128-131 for entry but the cell line used was derived from a choriocarcinoma and adapted to form monolayers. These cells may have lost cell surface receptors required for gH/gL/UL128-131 mediated entry during the transformation or the tissue culture adaptation process. We further observed, using a subset of mucosal epithelial cell lines, that antibodies to epitopes within the gH/gL/UL128-131 complex could block entry. These results support the hypothesis that a vaccine that elicits epithelial entry-specific neutralizing responses in mucosal secretions may provide sterilizing immunity.

In order to develop a vaccine focusing on epithelial neutralizing antibodies, the immune response at mucosal sites following natural infection needs to be better understood. Mucosal epithelial cells provide the first line of defense against CMV infection because initial exposure occurs at mucosal sites such as the oral or genital mucosa.[3] In the context of women with children in daycare, CMV transmission from child to mother is most likely occurring via the oral route through exposure to CMV in a child's saliva, urine, and tears. Therefore, in this highly susceptible population the immune response in the oral mucosa, particularly the salivary antibody response, may be critical for protection against CMV infection.

The salivary antibody response to CMV is poorly characterized. A single study completed in Japan in 1980 found that most CMV-infected infants had fibroblast entry neutralizing antibodies in their saliva.[110] However, in the current study neither epithelial nor fibroblast entry neutralizing activities were detectable in frozen saliva samples from children aged 2-24 months. Several factors may contribute to discrepancies between the two studies including differences in demographics, subject's health, sample collection, or sensitivities of the assays. The Japanese study analyzed fresh saliva samples from infants in a plaque reduction assay on fibroblast cells while the current study analyzed samples frozen approximately four years prior to use in a fluorescent-based neutralization assay on epithelial and fibroblast cells. The neutralization assay in the current study is based on measuring GFP expression in infected cells, which for technical reasons requires a higher viral dose than a plaque reduction assay. Hence, the neutralization assay used in the current study mixed samples with 5000 PFU of virus while the Japanese study used only 800 PFU of virus. Since the Japanese study determined neutralizing activity with six-fold less virus it may have detected neutralizing activity present at

levels below the limit of detection of our assay. The low sensitivity of the fluorescent-based neutralization assay is necessary because the more sensitive method of visually detecting viral infection by cytopathic effect does not work with epithelial cells.

Antibodies to gB are present in the saliva of CMV-infected infants at levels high enough to be detected by ELISA, but these antibodies are not necessarily neutralizing.[50] Lack of strong neutralizing antibodies in the saliva of children under two years of age was not unexpected because infants do not develop mature antibody responses during the first year of life. Unfortunately, serum samples from these children were not available for comparison. Infants do not start producing antibody until they are approximately six months old, and initial antibodies are not mature.[36] Additionally, IgA and IgG are not present at appreciable levels in the saliva until tooth eruption, which starts around six months.[123] Differences in the length of CMV shedding between young children (average of 18 months) and adults (at most a few weeks) may be due to immunologic immaturity of young children. While it is not known whether the children in this study were shedding virus at the time of saliva collection, urine was collected from seven children 3-4 weeks following saliva collection. All seven urine samples were CMV culture positive. Suggesting that many if not most of the children in this study were shedding CMV at the time of saliva collection. It would be interesting to follow young children over time to determine if the appearance of salivary neutralizing antibodies correlates with cessation of viral shedding in either the urine or saliva. To address this issue a longitudinal study that enrolls children when they enter daycare and monitors shedding in the urine and saliva along with salivary epithelial entry neutralizing activity of the children for 1-2 years is being planned.

Salivas from 17 children and eight adolescents were negative for neutralizing activity yet positive for gB-specific IgG (indicating that all were CMV infected). In contrast, 50% of seropositive adults were positive for salivary neutralizing activity. This suggests either that the natural history of CMV infection in children and adolescents differs from adults, or that immunological immaturity of children or adolescents attenuates the salivary humoral response. Children may be more efficient transmitters of CMV because they shed virus in saliva but do not manifest salivary antibodies that inactivate the virus. The fact that the salivas from children and adolescents were frozen for several years prior to analysis should not have altered our results because all of the saliva samples from adults were frozen for at least a few weeks prior to analysis. These data need to be expanded upon with a large group of subjects over a wide variety of ages (infants, toddlers, adolescents, and adults) to determine if age is a factor in the development of salivary neutralizing antibodies.

The presence of gB specific antibodies (IgG, IgA, and sIgA) in the saliva of healthy adults and CMV vaccine recipients has been demonstrated. Wang et al. detected IgG to gB by ELISA in salivas from 100% of adults that were naturally infected or gB/MF59 immunized, but the neutralizing activity of these gB-specific antibodies was not determined. Levels of anti-gB IgG in saliva strongly correlated with serum levels, but were approximately 1,000-fold lower.[51] This is consistent with salivary IgG being passively transferred into oral secretions. Salivary sIgA to gB was detected in only 50% of subjects in both groups, implying that transudated IgG may be the CMV-reactive immunoglobulin most consistently present in saliva. Our data on neutralizing activities are consistent with these results. We observed a correlation between saliva and serum neutralizing titers, and the slope of the linear regression line was 1:627, similar to the 1:1000

saliva/serum ratio reported by Wang et al. for gB-specific IgG [51] and to reported IgG saliva/serum ratios of 1:800 to 1:1000.[48,124]

Assuming that salivary neutralizing activity is primarily IgG-mediated and that IgG transudated from plasma is present in saliva at concentrations ~1000-fold lower than serum, no neutralizing activities should be detected in saliva when serum titers are below ~1:2000, as the predicted salivary titers (<1:2) would be below the assay's limit of detection. Indeed, all adults with serum epithelial entry neutralizing titers below 1:1777 lacked epithelial entry neutralizing activity in their salivas, while fibroblast entry neutralizing activities were never detected in saliva, presumably because fibroblast-based titers in serum never exceeded 1:835. Similarly, salivas from Towne recipients were all negative for neutralizing activity, presumably because Towne recipients have low serum epithelial entry neutralizing activities.[62]

Conversely, salivary neutralizing titers correlated with serum titers, and mean serum titers for subjects with salivary activity were statistically higher than for those without. Three subjects, however, were inconsistent with this trend as they had serum epithelial entry neutralizing titers greater than 1:1777 yet lacked salivary neutralizing activity. This could be due to the sampling variability inherent to saliva collection, or to fluctuations in total salivary IgG levels.[47] Thus, while it appears that salivary neutralizing activity may be predominantly IgG-mediated, additional studies are needed to determine if actively secreted (sIgA) antibodies play a role in some subjects.

Three subjects in the adult group were clearly unique. They had the highest salivary neutralizing activities, their serum neutralizing and gB-ELISA titers were several fold above ranges typical for normal seropositives, and they had detectable serum IgG to UL130. These observations suggest that something unique about the viral exposure in these subjects resulted in

hyperstimulated humoral responses. One possibility is that these subjects were undergoing active CMV infections subsequent to primary infection. If so, high IgG avidity for all three subjects suggests that infection occurred at least 3 months prior. Alternatively, that all three subjects had young children (4-32 months) in daycare suggests the possibility that elevated humoral immunity may arise from frequent CMV exposure. Although their children were not cultured, in the past prevalence of viruria in children under two at this daycare has been as high as 70%. Consistent with this hypothesis, positivity for salivary neutralizing activity was more frequent among daycare mothers than non-daycare-associated adults. However, these findings should be considered preliminary due to the small size and demographic diversity of our study populations. A larger study is needed to confirm that mothers of children in daycare have elevated humoral responses to CMV.

While antibodies to various subcomponents of the gH/gL/UL128-131 complex can neutralize epithelial entry,[26,30,52,63] and even peptide epitopes from UL130 or UL131 can induce high titer epithelial entry neutralizing activity in rabbits (Peptide Vaccine Section) the neutralizing epitopes that dominate the human response to CMV infection remain poorly defined. In immunocompetent seropositive subjects ELISA or IFA reactivities to individually expressed UL128 or UL130 proteins are uncommon,[63] while immunoblot detection of antibodies to UL128, UL130, or UL131 in seropositive human sera has not been previously reported. Consistent with this, we found that most seropositive adults lacked immunoblot-reactive antibodies to UL128-131 proteins, although four subjects with unusually high neutralizing titers had detectable antibodies to UL130. These results suggest that natural infection produces weak humoral responses to the individual proteins such that antibodies are either absent or present at levels below the limits of detection for these assays, but in a few subjects humoral responses are

sufficiently robust that antibodies to UL130 can be detected. While this may imply that in humans UL130 is the better immunogen, it is also possible that for technical reasons (*e.g.*, better expression or membrane transfer of UL130) the UL130 assay is simply more sensitive. It is also not known whether the UL130 antibodies detected by immunoblot are neutralizing.

The apparent lack of antibodies to individual UL128-131 proteins in most seropositives suggests that the robust epithelial entry neutralizing responses induced by natural infections may target primarily conformational or multisubunit-dependent epitopes that are not adequately represented in the immunoblot, ELISA, and IFA assays discussed above. Consistent with this, of 17 monoclonal antibodies with potent epithelial entry neutralizing activities isolated from seropositive donors, only one reacted with an individual subunit; the others recognized epitopes formed by two or more subunits.[52] However, that these antibodies are exceptionally potent for virus neutralization[52] suggests another possibility -- that regardless of the nature of their epitopes, antibodies present below our limits of detection could, due to high potency, confer robust neutralizing activities.

The salivary neutralizing activities observed in this study (1:6 to 1:28) are likely to have biological relevance. Assuming inoculum titers under 10,000 pfu/ml (a conservative estimate, based on titers of urines from viruric daycare children; author's unpublished data) and inoculum volumes less than 10 µl (a conservative estimate for hand-to-mouth transmission), the typical exposure dose of CMV probably does not exceed 100 pfu. In our studies salivas with the highest neutralizing activities (1:21-1:28) diluted 1:4 (the lowest tested) nearly eliminated infectivity of 5000 pfu of CMV (only a few cells became infected, Figure 10). Undiluted, these salivas should be capable of fully neutralizing a 100 pfu inoculum. How to achieve these levels of salivary neutralizing activities through vaccination remains uncertain. If transudation is taken as the

primary mechanism, our results suggest that measureable salivary neutralizing activities may be achieved when serum epithelial entry neutralizing titers exceed ~1:2000, while higher and presumably more protective titers in the 1:20-1:30 range may require serum titers of 1:7,000 to 1:16,000. Future vaccine studies may provide the means to test these predictions and to determine if neutralizing activity in saliva correlates with protection.

Peptides as possible subunit vaccines

The antigen specificities of the epithelial entry neutralizing antibodies in sera from naturally infected people are unknown, but because both the gH/gL/UL128-131 complex and this high level of neutralizing activity are specific to epithelial cell entry, the antibodies that comprise the epithelial entry neutralizing activity presumably target gH/gL/UL128-131. A vaccine that incorporates gH/gL/UL128-131 epitopes to induce epithelial entry neutralizing activities might be effective at preventing viral acquisition through mucosal epithelia.

Little is known about the neutralizing epitopes within the gH/gL/UL128-131 complex, and of central importance for vaccine design, it remains uncertain whether conformational epitopes unique to the full gH/gL/UL128-131 complex will be required, or whether subunits or even peptides will be sufficient to elicit neutralizing activities comparable to natural infection. Some evidence suggests that neutralizing epitopes often require multi-subunit complexes. As discussed above a panel of 17 human monoclonal antibodies having potent neutralizing activities against epithelial entry predominantly recognize epitopes that require two or more subunits. Only one of the 17 antibodies reacted with an individual subunit.[52] In addition, the Towne virus expresses UL128 and UL131, but expression of UL130 is impaired by a C-terminal frame shift that alters the protein's stability and steady-state levels.[33] Yet, despite the presumed ability to

express UL128 and UL131 *in vivo*, the Towne virus does not elicit high titer neutralizing antibodies specific for epithelial entry.[62] This may be because the absence of UL130 results in retention of the remainder of the complex (gH/gL/UL128/UL131) in the endoplasmic reticulum and subsequent failure of this complex to traffic to the cell surface or become incorporated into virions.[22] Thus, for a live attenuated vaccine, UL128 and UL131 are not sufficient.

Alternatively, animal antibodies raised against individual UL128, UL130, or UL131 peptides or recombinant proteins do neutralize epithelial or endothelial cell entry, indicating that each subunit contains neutralizing epitopes.[26,30,63] However, potency of animal antisera relative to human immune sera has not been reported. We observed that peptide epitopes within UL130 or UL131 can elicit epithelial entry neutralizing activities comparable to those induced by natural infection when administered to rabbits using optimal adjuvants. This indicates that the gH/gL/UL128-131 complex contains at least two potent neutralizing epitopes that do not require multi-subunit complexes. While the anti-UL128 peptide serum did not neutralize, the peptide used to raise this serum contained an inadvertent isoleucine insertion, and although it retains epitopes sufficient for the antiserum to recognize the native protein,[32] the possibility remains that the isoleucine disrupts a neutralizing epitope. Moreover, as UL128, UL130, and UL131 are respectively 171, 235, and 129 amino acids long, significant regions of these proteins have not been evaluated and may contain additional neutralizing epitopes. Indeed, that at least two of the three peptides studied contain neutralizing epitopes suggests that there may be many more.

This data does not necessarily imply that a vaccine based on the peptide epitopes described here would be effective in humans. Development of such a vaccine would face several hurdles. *First*, the rabbit immunization protocol used here was designed to elicit maximal antibody

responses and cannot be recapitulated in humans. To achieve comparable antibody responses in humans it may be necessary to utilize alternative adjuvants, carriers, or vector systems that are being developed specifically to elicit robust responses to peptide epitopes.

Second, it is not known whether these particular epitopes are immunogenic in humans. Serum antibodies from naturally infected individuals detectable by ELISA (using bacterial expressed proteins) to UL128 and UL130 have been reported (bacterial expressed UL131 ELISA assays were not reported). 75% of pregnant women experiencing a primary CMV infection had detectable UL130 but not UL128 antibodies in this assay. In contrast, UL128-reactive antibodies detected by ELISA were more common in immune-competent individuals and transplant recipients. The number of subjects used in this study was small (n=10 immune-competent, n=4 pregnant with primary infection, and n=4 transplant recipients) but the data suggest that UL128 and UL130 are immunogenic following natural CMV infection. This study also found immune-fluorescence serum reactivity to cells transfected with UL128, UL130, and UL131 in 3 out of 16 sera tested, suggesting that conformational epitopes to a UL128-131 complex also occur following natural infection.[63] Of the 17 potently neutralizing monoclonal antibodies to the gH/gL/UL128-131 complex that have been identified, only a single non-conformational or single subunit antibody was identified. The remaining antibodies required expression of at least two proteins in the pentameric complex, with antibodies recognizing combination of UL130 with UL131 most common.[52] Additionally, the neutralizing activity of saliva section describes the first report of UL130 antibodies detectable by western blot. Only 4 of 16 subjects tested had detectable UL130-reactie serum antibodies. However, vaccination may be more effective than infection at eliciting anti-peptide antibody responses, and, given that monoclonals that neutralize

this entry pathway are exceedingly potent,[52] it is possible that low antibody levels could confer significant neutralizing activities.

Third, the UL130 peptide exhibits strain heterogeneity and thus antibodies to this epitope may not cross-neutralize all CMV strains. Thus, while it may be possible to overcome these obstacles using novel immunization strategies and inclusion of additional or alternative epitopes, the more instructive implication of our results for vaccine development is that peptide or single subunit immunogens have the potential to produce high titer epithelial entry neutralizing responses, and hence, representation of complex conformational epitopes may not be necessary.

Epithelial entry mediator DNA vaccines

The first-generation UL128, UL130, UL131 DNA vaccines constructed used the strain TR sequences because the TR strain of CMV contains wild-type copies of each gene. Immunized mice did not produce epithelial entry neutralizing activities or antigen-specific antibodies in their sera. These initial data suggested that either: (A) DNA vaccination did not result in proper expression and processing of these proteins; or (B) antibodies to vaccine-specific antigens were not induced at sufficient levels. To determine if the DNA vaccine approach induced low level or inconsistent antigen-specific antibody responses, the vaccine plasmids were redesigned to increase their expression. Second-generation vaccines used codon-optimized sequences from the Towne genome for UL128, repaired UL130, and UL131, which are more representative of clinical strain sequences in GenBank than the TR strain.

The second-generation vaccine was more immunogenic since it induced antigen specific antibodies in 8/10 mice vaccinated with UL130 and epithelial entry neutralizing activity in 2/10 mice in the same group (Figures 27 and 29). Neutralizing activity correlated with UL130

antibodies measured in densomiteric units from western blots. These data indicate the majority of UL130 vaccinated mice made vaccine-specific antibodies but the resulting neutralizing activities remained below the level of detection of our neutralizing assay. The inconsistency in vaccine-specific antibody production seen here may be due to several factors. *First,* induction DNA vaccine-specific antibodies can be highly variable depending on the delivery method and target tissue.[125] *Second,* injecting the rectus femoris muscle is technically difficult. It is possible that the two mice with epithelial neutralizing titers of 1:150 and 1:214 received three effective immunizations while the six mice with UL130 antibodies but no neutralizing activity only received one or two effective immunizations and mice without UL130 antibodies received one or no effective immunizations. *Third,* mouse-to-mouse variation may be an issue. Although Balb/c mice are presumably genetically identical, different mice could react to different epitopes due to the genetic recombination involved in B cell development. Based on western blot signals not specific to the antigen tested, there is clearly some heterogeneity between mice (Figures 23 and 29). *Fourth,* some UL130 antibodies may be to non-neutralizing epitopes.

Lack of immunogenicity of the UL128 and UL131 DNA vaccines may be because these proteins do not induce strong immune responses, but may also arise from instability of these proteins when expressed individually. UL128 and UL131 are retained and degraded in the ER in the absence of the other proteins in the UL128-131 complex,[32] thus it is possible that UL128 and UL131 are degraded too quickly to be processed for antigen presentation to CD4 cells. Theoretically, co-immunization with all three plasmids should overcome the instability of individual immunizations, but in practice this did not improve immunogenicity. In fact, immunization with UL130 in combination with UL128, or UL128 and UL131 reduced the

immunogenicity of UL130 compared to solo vaccination, since no mice in these groups had serum neutralizing activity but 2/8 mice in the UL130 vaccinated group had activity. One mouse in the UL128+UL130, two mice in the UL130+UL131, and one mouse in the UL128+UL130+UL131 groups had borderline activity in the 1:30 screen but on detailed examination lacked epithelial entry neutralizing activity over background levels of pre-immune sera. Six mice in the UL130+UL131 group had detectable UL130 antibodies and one mouse had UL131 antibodies detectable by western blot. The UL128+UL130+UL131 group also had six mice with detectable UL130 antibodies. It is possible that these mice have UL130 antibodies with neutralizing activity below the level of detection, similar to the 6/10 mice in the UL130 group. Reduced levels of UL130 antibody in the combination groups are also expected due to reduced dosage (50 μg in pairs and 33 μg in triple) of individual DNAs. Co-immunization of multiple plasmids can also reduce expression of each protein due to dilution of available transcription factors. When UL130 is immunized alone it is the only plasmid competing for transcription factors but when all three plasmids are injected each plasmid may be transcribed at lower levels.

The data from the second-generation DNA vaccine study suggest that lack of a detectable antigen-specific response in the pilot DNA vaccine study was due to insufficient antibody production. The codon-optimization and the use of Towne sequences did not significantly alter the UL128, UL130, or UL131 *in vitro* expression, but *in vivo,* vaccination resulted in detectable UL130 antibodies in 80% of immunized animals. This may be due to better formulation and immunization technique (these immunizations were conducted by experienced personal at Vical Inc.), improved western blot antigens, or improved *in vivo* expression and presentation of the

second-generation UL130 vaccine. It is possible that in both studies most mice had epithelial entry neutralizing activities but they were below the level of detection of our assay. However, this may be of minor importance, given our goal of achieving highly robust activities.

Impact on future vaccine design

Although theoretically compelling, the premise that epithelial entry neutralizing antibodies can protect against infection is supported mainly by evidence that naturally acquired humoral immunity, which has high epithelial entry neutralizing activity, provides clinical benefits,[15,126,127] whereas experimental vaccines that induce weak epithelial entry neutralizing responses (compared to natural infection) [62] provide either partial[128] or no protection against primary infection.[79] Thus, the use of seropositive sera as a benchmark for evaluating immunogens is somewhat arbitrary; neutralizing activities comparable to those found in seropositive sera may not provide adequate protection, and while higher levels may be achievable and might enhance protection, other factors, such as cellular immunity or antibodies that neutralize fibroblast entry, may also be important. Ultimately, the importance of epithelial entry neutralizing antibodies for CMV vaccine protection may only be resolved through clinical trials of candidate vaccines that elicit neutralizing activities equivalent or superior to natural infection. The data presented here may aid in development of such candidate vaccines.

The fact that immunization with gB alone mimics naturally acquired immunity when sera are assayed on fibroblast cells supports the historical use of gB-focused vaccines, but the large differential between the gB/MF59 vaccine and natural infection with respect to epithelial entry neutralizing activity demonstrates that gB as a solo immunogen is not ideal. The addition of subunits that target epithelial cell entry to a gB vaccine may drastically increase the protection

offered. CMV entry mediators UL128, UL130, and UL131 are very attractive candidates for a subunit vaccine because these proteins are necessary for entry into epithelial cells but not fibroblast cells and neutralizing epitopes appear to be far more potent. This is in contrast to gB which is necessary for entry into both cell types but epitopes may be up to 1,000-fold less potent.[26,29] Vaccination with proteins that will induce epithelial entry-specific neutralizing antibodies should more closely mimic natural infection than vaccines such as gB/MF59 and Towne, which lack potent epithelial entry mediator targets.

Future studies comparing induction of mucosal immunity and protection from the gB/MF59 vaccine would be very informative. It is possible that gB/MF59 recipients that did not acquire CMV had high salivary epithelial entry neutralizing activities and that vaccine recipients that developed CMV disease had low or absent salivary epithelial entry neutralizing activity. Additionally, since the gB/MF59 vaccine induces very high gB antibodies but does not induce epithelial entry neutralizing activity similar to that of natural infection (but higher than Towne), addition of an epithelial entry mediator target to the vaccine could increase efficacy. The apparent synergy in epithelial neutralizing activity seen when anti-peptide sera to UL130 and UL131 are mixed suggests that addition of UL130 to a gB vaccine could produce a similar synergistic increase in epithelial neutralizing activities. A gB/UL130/MF59 vaccine has the potential to induce high fibroblast (from gB) and epithelial (from gB and UL130) entry neutralizing titers and the presence of increased epithelial entry neutralizing activity may greatly increase the protection provided by the vaccine.

Live attenuated viruses also offer the ability to induce antibodies to block entry into both epithelial and fibroblast cells. The Towne vaccine, which has a positive safety profile but

induces minimal protection, has a frame shift mutation in the *UL130* gene that leads to rapid degradation of UL130[33] and prevents expression of gH/gL/UL128-131 on the virion surface (Cui et al., submitted). Repair of the *UL130* mutation results in expression of the complex on the virion surface. A live attenuated UL130-repaired Towne vaccine should induce epithelial entry neutralizing activity in addition to the fibroblast entry neutralizing activity that Towne vaccine is known to induce. The chimeric Towne/Toldeo vaccine also has the potential to induce epithelial entry neutralizing activity greater than that induced by Towne. Although currently uncertain, two of the four chimeric viruses may encode wild-type copies of the *UL128*, *UL130*, and *UL131* genes. These viruses did not boost pre-existing immunity,[80] but they have not yet been tested in CMV naïve individuals. These vaccines are scheduled to be tested in a dose-escalation trial in CMV seronegative males. Once the vaccines are available they can be sequenced to determine if wild-type copies of *UL128-131* are present and the epithelial tropism of each virus can be determined, and immunogenicity can be compared to Towne.

The importance of fibroblast neutralizing activity *in vivo* is not known but it may play a role in protection. CMV vaccine design has historically focused on inducing fibroblast entry neutralizing titers, particularly to gB, because of the high level of gB antibodies found following natural infection. Neutralizing antibodies to gH are also produced following natural infection, but this protein cannot be properly expressed without gL.[5] It is possible that a gH/gL vaccine will induce more protective immune responses than gB. A vaccine representing the entire pentameric gH/gL/UL128-131 complex has the potential to induce neutralizing antibodies to block entry into both cell types because gH/gL-specific antibodies will target both epithelial and fibroblast entry, while UL128-131 antibodies will provide potent epithelial entry neutralizing

activity. Another more technically feasible approach would be a gH/gL/UL130 vaccine that would provide antibodies to target entry into both cell types.

A novel vaccine approach would be a subunit vaccine that induces epithelial but not fibroblast entry neutralizing activity. Since epithelial entry neutralizing activity is significantly higher than fibroblast entry neutralizing activity, absence of fibroblast entry neutralizing activity may not impact protective antibodies. Vaccination with peptides from UL130 and UL131 induces epithelial entry neutralizing activity in rabbit sera similar to that seen following natural infection. The peptides described here were not originally designed to be vaccine candidates and additional immunogenic peptides in UL128, UL130, and UL131 most likely exist. Future studies will involve screening each protein for immunogenic regions to develop additional peptide vaccine candidates.

The peptide immunization strategy used in this study cannot be translated for use in humans but other vaccination methods for peptide antigens are available. Virus-like particles (VLPs) are non-infectious, self-assembling proteins that have shown potential as peptide vaccine platforms. The hepatitis B core antigen (HBcAg) is a highly immunogenic VLP that has been tested in clinical trials with malaria and flu peptide inserts. Peptides engineered in HBcAg platform are expressed in *E. coli* and are highly immunogenic in animals. HBcAg is a 21-kDa polypeptide that spontaneously assembles into an icosahedral structure that is 36 nm in diameter. Each particle contains 240 HBcAg polypeptides. The protein contains a loop that is located on the outside of the assembled core particle with a highly immunogenic B cell epitope. Peptides of interest can be engineered into this loop to form a highly immunogenic VLP-based vaccine. Due to concerns of pre-existing immunity the HBcAg vaccine platform has been developed in rodent

HBcAg; woodchuck HBcAg is the most common.[129] The UL130 and UL131 peptides described here have been engineered into the duck and woodchuck hepatitis B core antigen (HBcAg) platforms. Once adequate amounts of protein are available all four constructs will be tested in mice for immunogenicity. We predict that these peptide-based vaccines will be immunogenic in small animals and easily translatable to clinical trials due to the safety and immunogenicity profile of this vaccine platform in previous clinical trials.[129]

UL130 is a potential candidate for an epithelial entry targeted vaccine. 80% of mice vaccinated with the UL130 second-generation DNA vaccine produced antigen-specific antibodies and 20% of these mice had epithelial entry neutralizing activity above 1:150. These titers are approximately ten-fold below natural infection. The DNA vaccine approach is inconsistent, and in mice may not be the best method to consistently induce high antigen-specific antibodies but may work better in larger animals such as rabbits and humans. KLH-conjugated UL130 peptide induced epithelial entry neutralizing titers that are about ten-fold above the DNA-vaccinated mice and in the range of natural infection, providing proof of concept that UL130 antibodies have the potential to reach neutralizing activities comparable to natural infection. Finally, UL130-specific antibodies are present in sera following natural infection indicating that vaccine-induced UL130 antibodies may be clinically relevant.

Immunization with the UL130 DNA vaccine or baclovirus-expressed UL130 protein has the potential to induce epithelial entry neutralizing activity similar to that of natural infection since this effect is seen following UL130 peptide immunization. Furthermore, the immunogenicity of a UL130 DNA vaccine, and possibly the UL128 and UL131 DNA vaccines, may be improved by use of a prime-boost strategy. Previous studies demonstrated that DNA

vaccines for MCMV and RhCMV provide an effective prime for an inactivated virion boost.[67,71] Future studies will involve a prime with the UL128, UL130, and UL131 DNA vaccines followed by an inactivated virion boost using a virus that is wild type for *UL128-131*. A virion boost may solve the inconsistency issue of the UL130 DNA vaccine. A second prime-boost strategy involves baclovirus expressed UL128, UL130, and UL131 protein subunits as a boost following DNA vaccine prime. DNA vaccination of mice with UL130 followed by inactivated virion or protein boost may be more effective than DNA prime with a trivalent DNA vaccine of UL128, UL130, and UL131 with either boost.

An alternative vaccine approach would be to add the UL130 DNA vaccine to the gB/pp65 DNA vaccine currently in clinical trials. The T cell response to UL130 has not been studied but since the anti-CMV repertoire is very broad it is possible that DNA vaccine induction of UL130-specific CD8 cells could augment the pp65-specific CD8 T cell response from the gB/pp65 DNA vaccine. Neutralizing activity of sera from gB/pp65 DNA vaccine recipients has not been published but based on the immunogens epithelial entry neutralizing activity is predicted to be lower than natural infection. However, the ability of antibodies to control disease in transplant recipients was demonstrated in a recent gB/MF59 trial that showed reduced CMV disease in vaccinated individuals.[65] Addition of epithelial entry mediator target UL130 to the vaccine may enhance epithelial entry neutralizing activity relative to the current vaccine. The gB/pp65 DNA vaccine could also be improved by addition of an inactivated virion expressing gH/gL/UL128-131 or UL130 protein boost. The gB/pp65 DNA vaccine primes the recipients' immune system for a Towne boost by decreasing the time to detectable immune response but the vaccine does not increase the median peak immune response compared to Towne alone.[87]

Addition of UL130 to the DNA prime and boosting with UL130-repaired Towne may increase the median peak immune response.

The RhCMV model has emerged as a critical tool in CMV vaccine development and is well suited to evaluation of vaccines targeting epithelial entry. Similar to human CMV, the RhCMV UL/b' region contains epithelial tropism genes, and viruses that contain full length UL/b' regions are more pathogenic *in vivo*. The RhCMV UL/b' region epithelial tropism genes have sequence homology to human CMV *UL128-131* genes and RhCMV strains with mutations in these genes cannot enter epithelial cells.[72,130] These features of the RhCMV UL/b' region make the testing of epithelial entry mediator targets against CMV infection in a animal model possible.

Additionally, RhCMV is shed in the saliva of seropositive monkeys. DNA prime/virion or MVA boost vaccine strategies in the RhCMV model reduce viral shedding in the saliva of vaccinated monkeys and decrease transmission of challenge virus to seronegative monkeys.[72] When seronegative monkeys are housed with seropositive monkeys they are highly susceptible to infection within 192 days, with a doubling of infected animals occurring every 49 to 56 days.[130] Therefore, co-housing experiments provide an authentic animal model of CMV transmission that can be used to determine if vaccination can reduce transmission. Vaccination of monkeys with epithelial entry mediator targets and determining if vaccination can reduce RhCMV transmission will be a valuable tool in CMV vaccine development.

Regardless of the vaccine being evaluated, our findings suggest sera and saliva should be evaluated for neutralizing activity on epithelial cells. Natural infection does not always provide sterilizing immunity to CMV so a protective vaccine needs to induce neutralizing activity stronger than that typically induced by natural infection. We recently identified highly immune individuals with serum neutralizing titers at least three-fold higher than mean natural infection titers and saliva neutralizing titers approximately two-fold higher than mean natural infection titers. It is possible that these individuals, all mothers of small children in daycare, have such high titers because they are chronically exposed to CMV in their children's urine, saliva, and/or tears. Future studies will enroll women at the time their children start daycare and follow their serum and saliva epithelial entry neutralizing activities over time while monitoring CMV shedding in their children. The results may correlate high maternal serum and saliva neutralizing activities in mothers with CMV shedding children.

These highly immune women provide a new benchmark for vaccine effectiveness because they demonstrate that extremely high neutralizing activities in serum and saliva are achievable. They also demonstrate that high serum neutralizing activities correlate with the presence of saliva neutralizing activity. This suggests that if high enough serum epithelial entry neutralizing titers are induced by vaccination, mucosal immunity in the form of saliva epithelial entry neutralizing activities will also be induced. Since CMV infection initially occurs predominately at the oral mucosa, vaccine-induced salivary epithelial entry neutralizing activity may be the key to a protective vaccine. Therefore, vaccines should strive to induce an immune response similar to the highly immune individuals described here which is measured as serum epithelial entry neutralizing titers >1:7,000 and saliva epithelial entry neutralizing titers >1:20.

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APPENDIX

Young children in daycare subject information

Sample Number	Date Saliva Collected	Age (months)	Saliva gB-IgG ELISA	Date urine collected	Urine culture[a]	Date Saliva Tested
2	2.21.08	10	+	n.a.[b]	n.d.[c]	6.7.10
11	2.28.08	9	+	n.a.	n.d.	6.7.10
27	3.17.08	3	+	n.a.	n.d.	6.7.10
31	3.26.08	7	+	n.a.	n.d.	6.7.10
35-R	11.21.08	10	+	n.a.	n.d.	5.10.10
36	4.3.08	12	+	n.a.	n.d.	6.7.10
49	6.5.08	6	+	n.a.	n.d.	6.7.10
51	6.12.08	2	+	n.a.	n.d.	6.7.10
57-N	6.12.08	12	+	10.15.08	+	6.7.10
59	6.19.08	5	+	n.a.	n.d.	6.7.10
60	6.19.08	8	+	n.a.	n.d.	6.7.10
78-N	8.14.08	15	+	9.18.08	+	6.7.10
82-N	8.14.08	15	+	9.18.08	+	6.7.10
84-N	8.14.08	3	+	9.18.08	+	5.10.10
99-N	9.17.08	10	+	12.1.08	+	5.10.10
100-N	9.17.08	11	+	12.9.08	+	6.7.10
113-N	11.6.08	20	+	12.15.08	+	6.7.10

[a] 3-4 weeks post saliva collection

[b] n.a. = not applicable

[c] n.d. = not determined

Adolescent subject information

Sample Number	Date Collected	Saliva gB-IgG ELISA	Date Saliva Tested
Ad 1	August 2008	+	6.18.10
Ad 4	August 2008	+	6.18.10
Ad 5	August 2008	+	6.18.10
Ad 6	August 2008	+	6.18.10
Ad 12	August 2008	+	6.18.10
Ad 13	August 2008	+	6.18.10
Ad 15	August 2008	+	6.18.10
Ad 18	August 2008	+	6.18.10
Ad 2	August 2008	-	6.18.10
Ad 3	August 2008	-	6.18.10

Women with children in daycare additional information

Sample Number	Date Collected	Child Age (months)	Date Serum and Saliva Tested
M1 (FMS 5)	8.23.10	11	9.17.10
M2 (FMS 6)	8.23.10	13	9.20.10
M3 (FMS 9)	8.23.10	Not given	9.27.10
M4 (FMS 11)	8.26.10	4 and 31	10.1.10
M5 (FMS 12)	8.26.10	12	10.1.10
M6 (FMS 15)	8.26.10	32	10.4.10
M7 (FMS 17)	8.26.10	19	10.11.10

Non-daycare associated adults additional information

	Serum		Saliva	
Sample Number	Date Collected	Date Tested	Date Collected	Date Tested
A1 (BM)	11.29.04	7.5.10	6.17.20	7.5.10
A2 (spec 1450)	6.18.09	7.5.10	4.21.10	7.5.10
A3 (WK)	7.9.96	7.5.10	6.17.20	7.5.10
A4 (spec 1469)	6.3.10	7.5.10	6.17.20	7.5.10
A5 (RL)	11.4.02	7.5.10	6.17.20	7.5.10
A6 (spec 1422)	2.14.08	7.9.10	4.21.10	7.9.10
A7 (spec 1342)	10.13.04	7.9.10	6.17.20	7.9.10
A8 (spec 1470)	6.3.10	7.9.10	6.17.20	7.9.10
A9 (FMS 10)	8.25.10	9.27.10	8.25.10	9.27.10

Towne vaccine recipients subject information

Sample Number	Date Collected	Months post-Vaccination	gB ELISA	Date Saliva Tested
25181	7.22.98	2	6400	7.16.10
25192	7.23.98	8	6400	7.16.10
25184	7.21.98	6	25600	7.16.10
25185	7.21.98	8	25600	7.16.10
27441	2.28.00	9	25600	7.16.10
27462	3.7.00	10	25600	7.16.10
27558	5.16.00	7	25600	7.16.10
27560	5.9.00	36	6400	7.16.10

CMV negative adults

Non-daycare associated adults information

	Serum		Saliva	
Sample Number	Date Collected	Date Tested	Date Collected	Date Tested
A10 (spec 1457)	1.26.10	7.9.10	6.17.20	7.9.10
A11 (spec 1471)	6.3.10	7.9.10	6.17.20	7.9.10
A12 (spec 1473)	4.21.10	n.d.	4.21.10	4.22.10
A13 (AM)	n.d.	n.d.	4.21.10	4.22.10

Adults with children in daycare infomation

		Serum		Saliva	
Sample Number	Child Age (months)	Date Collected	Date Tested	Date Collected	Date Tested
M8 (FMS 1)	13	8.23.10	9.3.10	8.23.10	9.3.10
M9 (FMS 2)	7	8.23.10	9.3.10	8.23.10	9.17.10
M10 (FMS 3)	4	8.23.10	9.3.10	8.23.10	9.17.10
M11 (FMS 4)	6	8.23.10	9.3.10	8.23.10	9.17.10
M12 (FMS 7)	18	8.23.10	9.20.10	8.23.10	9.20.10
M13 (FMS 8)	11	8.23.10	9.20.10	8.2.310	9.27.10
M14 (FMS 13)	8	8.26.10	10.1.10	8.26.10	10.4.10
M15 (FMS 14)	Not given	8.26.10	10.4.10	8.26.10	10.4.10
M16 (FMS 16)	7	8.26.10	10.11.10	8.26.10	10.11.10
M17 (FMS 18)	5	8.26.10	10.11.10	8.26.10	10.15.10
M18 (FMS 19)	47	8.26.10	10.15.10	8.26.10	10.15.10
M19 (FMS 20)	45	8.26.10	10.15.10	8.26.10	10.15.10

Towne vaccine placebo recipients information

Sample Number	Date Collected	Months post- mock Vaccination	Date Saliva Tested
25182	7.22.98	7	7.16.10
25202	7.28.98	4	7.16.10

Subjects in Figure 16 panel information

Sample Number	Date Collected	gB ELISA	Epithelial Neutralization	Date Serum Tested
Pos 1 (W706)	10.05.05	1:102400	1:1007	12.10.10
Pos 2 (spec1468)	6.3.10	1:102400	1:1396	12.10.10
Pos 3 (spec 1474)	7.14.10	1:102400	1:1943	12.10.10
Pos 4 (spec 1476)	7.14.10	1:25600	1:2230	12.10.10
Pos 5 (W534)	4.18.03	1:25600	1:2729	12.10.10
Pos 6 (spec 1467)	6.3.10	>1:102400	1:3118	12.10.10
Neg 1 (DCP 1466)	4.2.10	<1:1600	<1:10	12.10.10
Neg 2 (spec 1466)	4.14.10	<1:1600	<1:10	12.10.10

VITA

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Background	
Born in Manhasset, NY	October 11, 1981

Education	
Virginia Commonwealth University, Richmond, VA MD/Ph.D. in Microbiology and Immunology Dissertation: "CMV vaccine development based on epithelial entry mediators UL128, UL130, and UL131"	2005-Present
Worcester Polytechnic Institute, Worcester MA B.S. in Biology and Biotechnology, Minor in International Studies Graduated with High Distinction	1999-2003

Research Experience	
Virginia Commonwealth University, Richmond, VA Department of Pediatrics Graduate Student Principal Investigator: Michael McVoy, PhD	2009-Present
Virginia Commonwealth University, Richmond, VA Department of Microbiology and Immunology Graduate Student Principal Investigator: Anthony Nicola, PhD	2007-2009
University of Massachusetts Medical School, Worcester, MA Department of Pathology Research Associate	2003-2005

Principal Investigator: Liisa, Selin, MD

Worcester Polytechnic Institute, Worcester, MA — 2002-2003
Department of Biology and Biotechnology
Undergraduate Research Thesis
Principal Investigator: David Adams, PhD

University of Massachusetts Medical School, Worcester, MA — Summer 2001
Cancer Center
Undergraduate Research Assistant
Principal Investigator: Peter Newburger, MD

Teaching Experience

Virginia Commonwealth University School of Medicine, Richmond, VA — Fall 2005
Teaching Assistant in Anatomy

Worcester Polytechnic Institute, Worcester, MA — 2000-2003
Peer Writing Tutor, WPI Writing Center

Volunteer Experience

Richmond SPCA Foster Parent, Richmond, VA — 2011-Present

Firehouse Theatre, Richmond, VA — 2010-Present

Virginia Commonwealth University School of Medicine, Richmond, VA — 2005-2006
Smile Program Volunteer

University of Massachusetts Medical Center, Worcester, MA — 2001-2004
Department of Volunteer Services
Pediatric In-patient Unit 500+ hours

Worcester Polytechnic Institute, Worcester, MA — 2002-2003
EMS Squad

Worcester Polytechnic Institute, Worcester, MA — 1999-2003
Hoop Dreams Mentoring Program
Program Coordinator — 2002-2003

SUNY Stony Brook Hospital, Stony Brook, NY — 1998-2000
Department of Volunteer Services
Pediatric In-patient Unit 300+ hours

Awards and Honors

Tau Beta Pi
National Engineering Honors Society
Inducted December 2002

ASCI/APP Travel Award from 2009 APSA Meeting, Chicago, IL

Professional Affiliations

Richmond Academy of Medicine	2005-Present
Medical Society of Virginia	2005-Present
American Medical Association	2005-Present
Tau Beta Pi	2002-Present

Presentations

Poster at the 13th International CMV/Beta Herpesvirus Workshop Nuremberg, Germany Saccoccio FM, Gallagher MK, Adler SP, and McVoy MA. 2011. Neutralizing activity of saliva: a new vaccine benchmark?	May 2011
Poster at the 13th International CMV/Beta Herpesvirus Workshop Nuremberg, Germany Cui XH, Saccoccio FM, Smith L, Hartikka J, Adler SP, and McVoy MA. 2011. A DNA prime/inactivated virion boost vaccine strategy targeting epithelial entry neutralizing responses	May 2011
Oral Presentation at Infectious Disease Research Conference, Virginia Commonwealth School of Medicine, Richmond, VA Saccoccio FM, Gallagher MK, Adler SP, and McVoy MA. 2011. The role of mucosal immunity in CMV infection and vaccine development	April 2011
Oral Presentation at the 2nd Annual Virginia Herpesvirus Symposium, Virginia Beach, VA Saccoccio FM, Gallagher MK, Adler SP, and McVoy MA. 2011. Detection of salivary neutralizing antibodies an serum UL130 antibodies in cytomegalovirus immune adults	April 2011
Oral Presentation at the 1st Virginia Herpesvirus Symposium, Virginia Beach, VA Saccoccio, FM and McVoy, MA. 2010. Importance of epithelial	May 2010

cells for developing a CMV vaccine.

Poster at VCU Women's Health Day, Richmond, VA — April 2010
Saccoccio FM, Cui XH, and McVoy MA. 2010. Development of a Cytomegalovirus vaccine using viral proteins UL128-131

Poster at the 5th Annual Meeting of the American Physician Scientists Association, Chicago, IL — April 2009
Saccoccio FM and Nicola AV. 2009. Vesicular trafficking of HSV during viral entry

Poster at the 25th Annual Daniel T. Watts Symposium, Virginia Commonwealth University, Richmond, VA — 2008
Saccoccio FM and Nicola AV. 2008. Vesicular trafficking of HSV during viral entry

Publications

Saccoccio FM, Gallagher MK, Adler SP, McVoy MA. Neutralizing activity of saliva against cytomegalovirus. Submitted, Clinical and Vaccine Immunology May 2011.

Saccoccio F, Sauer A, Cui X, Armstrong A, Habib E, Johnson D, Ryckman B, Klingelhutz A, Adler S, McVoy M. 2011. Peptides from Cytomegalovirus UL130 and UL131 proteins elicit epithelial entry neutralizing responses comparable to natural infection and capable of neutralizing viral entry into mucosal epithelial cells. Vaccine 29 (15): 2705-11 PMID: 21310190

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Cornberg M, Sheridan BS, Saccoccio FM, Brehm MA, Selin LK. 2007. Protection against vaccinia virus challenge by CD8 memory T cells resolved by molecular mimicry. J Virol 81 (2): 934-44. PMID: 17019318.