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(54) **Title:**
CYTOMEGALOVIRUS VACCINES AND METHODS OF PRODUCTION

(57) **Abstract:**
CYTOMEGALOVIRUS VACCINES AND METHODS OF PRODUCTION Abstract Methods of increasing diversity in cytomegalovirus vaccines through the selection of cell type in which the virus is propagated, and the use of cytomegalovirus produced by those methods in the development of vaccine compositions, are disclosed. Vaccine compositions comprising CMV isolated from epithelial cells are also disclosed. Fig. 1

CYTOMEGALOVIRUS VACCINES AND METHODS OF PRODUCTION

Abstract

Methods of increasing diversity in cytomegalovirus vaccines through the selection of cell type in which the virus is propagated, and the use of cytomegalovirus produced by those methods in the development of vaccine compositions, are disclosed. Vaccine compositions comprising CMV isolated from epithelial cells are also disclosed.

Fig. 1

CYTOMEGALOVIRUS VACCINES AND METHODS OF PRODUCTION

This claims benefit of U.S. Provisional Application No. 60/998,426, filed October 10, 2007, the entire contents of which are incorporated by reference herein.

Pursuant to 35 U. S. C. §202(c), it is acknowledged that the United States government may have certain rights in the invention described herein, which was made in part with funds from the National Institutes of Health under Grant Nos.: CA85786, CA82396, AI54430 and GM71508.

FIELD OF THE INVENTION

The invention relates generally to the field of vaccine development. More specifically, the invention relates to methods of increasing diversity in cytomegalovirus vaccines through the selection of cell type in which the virus is propagated, and to the use of cytomegalovirus produced by those methods in the development of vaccine compositions.

BACKGROUND OF THE INVENTION

Various publications, including patents, published applications, technical articles and scholarly articles are cited throughout the specification. Full citations for publications referenced by numbers in parentheses or otherwise not cited fully within the specification are set forth at the end of the specification.

Cytomegalovirus (CMV) is a herpes virus classified as being a member of the beta subfamily of herpesviridae. According to the Centers for Disease Control and Prevention, CMV infection is found fairly ubiquitously in the human population, with an estimated 40-80% of the United States adult population infected. The virus is spread primarily through bodily fluids, and is frequently passed from pregnant mothers to the fetus or newborn. In most individuals, CMV infection is latent, although virus activation can result in high fever, chills, fatigue, headaches, nausea, and splenomegaly.

Although most human CMV infections are asymptomatic, CMV infections in immunologically immature or immunocompromised individuals, such as newborns, HIVpositive patients, allogeneic transplant patients and cancer patients, can be particularly problematic. CMV infection in such individuals can cause severe morbidity, including pneumonia, hepatitis, encephalitis, colitis, uveitis, retinitis, blindness, and neuropathy, among other deleterious conditions. In addition, CMV is a leading cause of birth defects. At present, there is no cure or preventive vaccine for CMV infection.

The entry of herpesviruses into cells is a complex process initiated by adsorption and receptor binding and followed by fusion of the virus envelope with a cell membrane. Fusion occurs at cither the plasma membrane or an endosomal membrane. For instance, Epstein-Barr virus (EBV) enters primary B cells via receptor-mediated endocytosis (1, 2), yet it infects epithelial cells or transformed B cells by fusion of the virion envelope with the plasma membrane (1). Herpes simplex virus fuses with the plasma membrane of some cell types, but enters others by endocytosis (3-6). Human cytomegalovirus (HCMV) infects multiple cell types in vivo, including epithelial cells, endothelial cells and fibroblasts (7). It fuses with the plasma membranes of fibroblasts (8), but enters retinal pigmented epithelial cells and umbilical vein endothelial cells via endocytosis (9, 10).

The mechanism by which herpesviruses 'choose' their route of entry remains unclear. It is generally assumed that entry pathways are mainly determined by the host cell, but there is precedent for tropic roles of virion glycoproteins (11). EBV virions contain two gH complexes, gH/gL and gH/gL/gp42 (12, 13), which have mutually exclusive functions (11). Fusion with the plasma membrane of B cells is mediated by gH/gL/gp42 (14-16), but entry into epithelial cells is triggered by gH/gL (11, 12, 17). The cell type in which EBV is produced can alter its tropism. B-cell-derived EBV virions contain less gH-gL-gp42 than epithelial-cell-derived virions. As a result, B-cell-generated virus is more infectious for an epithelial cell and epithelial cell-derived virus is B cell tropic (18).

HCMV also encodes two gH/gL complexes: gH/gL/gO and gH/gL/pUL128/pUL130/ pUL13 1 (19, 20). The gO-containing complex is sufficient for fibroblast infection, whereas the pUL128/pUL130/ pUL13 1 -containing complex is required to infect endothelial and epithelial cells (19-21). The AD 169 laboratory strain contains only the gH/gL/gO complex in its virions (19). The absence of the second gH/gL complex is responsible for the loss of epithelial and endothelial cell tropism in HCMV laboratory strains (19-22).

US 20050064394 describes the systematic analysis of the Towne strain of a human CMV genome to identify 45 viral ORFs reported to be essential for viral replication and characterized 115 growth-dispensable viral genes. Also described are temperance factors that repress CMV replication in a cell type-specific basis. A cytomegalovirus with a deletion of one or more ORFs is suggested for use as a vaccine.

US 6,471,965 describes a combination of Herpes Simplex Virus 1, Herpes Simplex Virus 2, Herpes Simplex Virus 6, Human Cytomegalovirus and Epstein-Barr Virus for use as a vaccine.

US 4,058,598 describes attenuation of CMV by serial passage in human tissue culture cells for use in a vaccine.

There is a need for variety and diversity of CMV vaccines, and for effective means to control the spread and activation of the virus, particularly in immunocompromised individuals and pregnant women. The present invention addresses that need.

SUMMARY OF THE INVENTION

One aspect of the present invention features a method of making a cytomegalovirus (CMV) vaccine. The method comprises propagating strains or isolates of CMV in cultured cells of a selected cell type, thereby producing a cell type-conditioned CMV, and producing a CMV vaccine from the cell type-conditioned CMV. In certain embodiments, the CMV strain or isolate is a human CMV (HCMV) strain or isolate. A wide variety of cell types are suitable for the method, including but not limited to epithelial cells, endothelial cells, fibroblasts, neuronal cells, smooth muscle cells, macrophages, dendritic cells and stromal cells. In a specific embodiment, the selected cell type is an epithelial cell.

The aforementioned method can further comprise producing the cell type-conditioned CMV in two or more different selected cell types and combining those CMV to produce the CMV vaccine. Alternatively or additionally, the method comprises providing two or more CMV strains or isolates, growing each of the strains or isolates in the cultured cells comprising the selected cell type or two or more different selected cell types, and combining all the CMV produced therefrom to make the CMV vaccine.

In certain embodiments, the method comprises producing a live attenuated CMV vaccine. In other embodiments, it comprises producing an inactivated or killed CMV vaccine. In still other embodiments, it comprises producing combination vaccines comprising one or more live attenuated viruses, inactivated viruses and other immunogenic components, e.g., immunogenic CMV proteins and peptides, and the like.

CMV vaccines produced by the aforementioned methods are also within the scope of the present invention.

Another aspect of the invention features kit for practicing the methods of the invention. Such kits typically include a package in which is contained one or CMV strains or clinical isolates, cultured cells of one or more selected cell types, and instructions for using the cultured cells and the CMV strains or isolates to produce cell type-conditioned CMV for use in a CMV vaccine.

Another aspect of the invention features a vaccine composition comprising a cytomegalovirus (CMV) population or virion components thereof, admixed with a suitable pharmaceutical carrier or adjuvant, wherein the CMV population is isolated from an cultured cells of a selected cell type. In one embodiment, the selected cell type is an epithelial cell type. In one embodiment, the vaccine composition comprises HCMV.

In various embodiments of the vaccine composition, the CMV population isolated from epithelial cell cultures is characterized by one or more features in subsequently infected host cells including but not limited to: (a) entry into the host cells by fusion with host cell plasma membranes; (b) greater virion-mediated cell-cell fusion of the host cells as compared with an equivalent CMV population isolated from cultured fibroblasts; (c) accelerated virus growth in the host cells as compared with an equivalent CMV population isolated from culture fibroblasts: (d) elicitation of a cellular response involving changes in expression greater than or equal to 2.5 fold of about two thirds fewer genes than a response elicited by an equivalent CMV population isolated from culture fibroblasts at 10 hours post-infection: or (e) elicitation of a cellular response involving a change in expression of one or more genes as shown in Table 2 and Table 4 herein, the latter being represented by GenBank Accession Nos: AK094860, NM_145023, NM_133492, NM_001039580, NM_001004301, NM_001034, AI369525, AK123066, NM_005345, NM_020731, BC071797, NM_003414, NM_000800, NM_138467, AK090803, AL133118, NM_001165, BG001037, NM_024861, NM_001043, NM_016239, NM_001018084, NM_001037442, NM_017600, NM_022097, NM_175868, NM_032266, NM_003841, NM_005039, NM_145051, NM_004294, AW856073, NM_024050, AF085968, NM_080927, NM_022115, AK056703, NM_000808, NM_012377, NM_006793, NM_031466, NM_005185, NM_139173, BX360933, NM_016125, NM_002104, NM_032188, NM_004185, NM_004843 or NM_173550.

In certain embodiments, the vaccine composition comprises a CMV population or virion components thereof isolated from a cell culture of two or more different selected cell types. For instance, the CMV population may be isolated from as an epithelial cells and cells of another cell type, such as a fibroblast cell type. In other embodiments, the CMV population comprises two or more CMV strains or clinical isolates grown in the selected cell type. Certain embodiments can comprise a plurality of CMV strains or clinical isolates grown in cell cultures of a plurality of different cell types.

In one embodiment, the vaccine composition comprises a live attenuated CMV vaccine. In another embodiment, it comprises an inactivated CMV vaccine. In still other embodiments, the vaccine composition can be a combination vaccine comprising one or more strains of live attenuated virus or components thereof, inactivated virus or components thereof, and/or other immunogenic CMV peptides or proteins.

Another aspect of the invention features a method of immunizing an individual against CMV, comprising administering to the individual a CMV vaccine composition produced by the aforementioned methods and/or comprising the aforementioned features. In one embodiment, the individual to be immunized is a human.

Other features and advantages of the invention will be understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Kinetics of HCMV IE1 expression in ARPE-19 cells. (*A*) Infected cells (0.1 pfu/cell) were fixed at indicated times, and stained for IE1 (green in color photo, light gray in black and white photo), Sp100 (red in color photo, very dark gray in black and white photo) and DNA (blue in color photo, dark gray in black and white photo). (*B*) At various times after infection (0.1 pfu/cell), the percentage of IE1-expressing cells was quantified; results are shown on the graph.

Figure 2. Electron microscopic analysis of HCMV entry into ARPE-19 cells. *epi*BAD*r*UL131 or *fibro*BAD*r*UL131 particles (50 pfu/cell) were bound to cells at 4 °C and then allowed to internalize at 37 °C for 15 min. Representative images are displayed.

Figure 3. Effects of inhibitors of endosome acidification and virion source on HCMV entry into ARPE-19 cells. Experiments were performed in triplicate, and the number of positive cells in drug-treated relative to untreated cultures is reported. (*A*) Cells were pretreated with NH_4Cl or BFA for 1 h, inoculated with *epi*BAD*r*UL131 or *fibro*BAD*r*UL131 (1 pfu/cell) and stained for IE1 16 h later. (*B*) Cells were pretreated with 50 mM NH_4Cl or 40 nM BFA for 1 h, and then inoculated with BAD*r*UL131 (0.1 pfu/cell) or FIX*wt* (0.01 pfu/cell) produced in the indicated cell types and stained for IE1 16 h later.

Figure 4. Fusion from without of ARPE-19 cells induced by epithelial cell-derived virus. (*A*) Cells were inoculated with *epi*BAD*r*UL131 or *fibro*BAD*r*UL131 (20 pfu/cell) and then maintained in medium containing 200 μg/ml of PFA. Phase contrast images were taken at 16 h post infection. (*B*) A mixture of reporter and effector cells were infected by *epi*BAD*r*UL131 or *fibro*BAD*r*UL131 (20 pfu/cell) for at 4 °C for 1 h. The culture was then shifted to 37 °C for 6 h, after which relative luciferase activity was measured.

Figure 5. Effect of pUL130-specific neutralizing antibody on HCMV infection and entry. (*A*) Epithelial cell- or fibroblast-derived viruses were incubated with various concentrations of anti-pUL130, and residual infectivity was determined. (*B*) Epithelial cell- or fibroblast-derived virus particles were pretreated with anti-pUL130 at a final concentration of 20 μg/ml or with PBS, and then adsorbed to ARPE-19 cells at 4 °C for 1 h. The cells were

washed twice with cold PBS, and viral DNA associated with the cells was extracted to determine the relative numbers of particles attached to the cells. Alternatively, the cells were shifted to 37 °C for 2 h to allow the virus entry. Virions that did not penetrate the cells were removed by EDTA-trypsin treatment. Internalized viral DNA was subsequently quantified by real-time PCR.

Figure 6. Modulation of the ARPE-19 transcriptome by HCMV produced in epithelial cells versus fibroblasts. (*A*) Venn diagrams depict the distribution of differentially regulated genes at 6 h or 10 hpi with *epi*BAD*r*UL131 or *fibro*BAD*r*UL131(3 pfu/cell) relative to mock infection. (*B*) Changes in relative RNA levels assayed by real-time RT PCR. The genes tested are hydroxymethylbilane synthase (HMBS, NM_000190), GLI pathogenesis-related 1 (glioma) (GliPR, NM_006851), pentraxin-related gene, rapidly induced by IL-1 beta (PTX3, NM_002852), 2'-5'-oligoadenylate synthetase 3 (OAS3, NM_006187), interferon-induced protein 44 (IFI44, NM_006417), v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (relB, NM_006509), and ATP-binding cassette, sub-family C (CFTR/MRP), member 3 (MRP3, NM_003786).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Various terms relating to the methods and other aspects of the present invention are used throughout the specification and claims. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used. It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

Definitions:

As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a cell" includes a combination of two or more cells, and the like.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, more preferably ±5%. even more preferably ±1%, and still more preferably ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The terms "amplifying," "propagating," and "growing," or "amplification," "propagation," and "growth" are used interchangeably herein to refer to the general process of introducing virus into cultured cells or infecting cells with virus under conditions permitting the virus to replicate and multiply within the cells, in accordance with methods well known to virologists and medicinal biologists. In particular, these terms are used herein to refer to the step of the inventive method in which the CMV is "conditioned" by propagation on a selected cell type, as the step prior to using the conditioned CMV for the production of a vaccine.

"Biomolecules" include proteins, poplypeptides, nucleic acids, lipids, polysaccharides, monosaccharides, and all fragments, analogs, homologs, conjugates, and derivatives thereof.

"Cell culture" refers generally to cells taken from a living organism and grown under controlled conditions ("in culture" or "cultured"). A "primary cell culture" is a culture of cells, tissues, or organs taken directly from an organism(s) before the first subculture. A "cell line" is a population of cells formed by one or more subcultivations of a primary cell culture.

A "coding region" of a gene consists of the nucleotide residues of the coding strand of the gene and the nucleotides of the non-coding strand of the gene which are homologous with or complementary to, respectively, the coding region of an mRNA molecule which is produced by transcription of the gene.

A "coding region" of an mRNA molecule also consists of the nucleotide residues of the mRNA molecule which are matched with an anti-codon region of a transfer RNA molecule during translation of the mRNA molecule or which encode a stop codon. The coding region may thus include nucleotide residues corresponding to amino acid residues which are not present in the mature protein encoded by the mRNA molecule (*e.g.*, amino acid residues in a protein export signal sequence).

The terms "conditioned virus," "cell type-conditioned virus," "conditioned CMV" or "cell type-conditioned CMV" refer to CMV that has been propagated in a selected cell type prior to its use in vaccine production, in accordance with the methods described herein. These terms are intended to be analogous to the term "conditioned medium," which describes culture medium in which a particular cell type or cell line has been grown and then removed, and which contains components or factors produced by the cells, thereby altering the functionality of the medium. For purposes of the present application, the term "conditioned virus" similarly refers to virus that has been grown in a selected cell type and then removed from those cells, wherein the virus thereafter exhibits one or more altered functional features resulting from its growth in that cell type.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Effective amount" or "therapeutically effective amount" are used interchangeably herein, and refer to an amount of a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result. Such results may include, but are not limited to, the inhibition of virus infection as determined by any means suitable in the art.

As used herein "endogenous" refers to any material from or produced inside an organism, cell, tissue or system. "Exogenous" refers to any material introduced from or produced outside an organism, cell, tissue or system.

The term "expression" as used herein is defined as the transcription and/or translation of a particular nucleotide sequence driven by its promoter.

As used herein, "immunization" or "vaccination" are use interchangeably herein and are intended for prophylactic or therapeutic immunization or vaccination. "Therapeutic vaccination" is meant for vaccination of a patient with CMV infection.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell. Unless it is particularly specified otherwise herein, the proteins, virion complexes, antibodies and other biological molecules forming the subject matter of the present invention are isolated, or can be isolated.

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether *in vitro* or *in situ*, that can be infected with CMV. In certain non-limiting embodiments, the patient, subject or individual is a human.

"Parenteral" administration of an immunogenic or vaccine composition includes, *e.g.*, subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric "nucleotides." The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning and amplification technology, and the like, and by synthetic means.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

"Pharmaceutically acceptable" refers to those properties and/or substances which are acceptable to the patient from a pharmacological/toxicological point of view and to the manufacturing pharmaceutical chemist from a physical/chemical point of view regarding composition, formulation, stability, patient acceptance and bioavailability. "Pharmaceutically acceptable carrier" refers to a medium that does not interfere with the effectiveness of the biological activity of the active ingredient(s) and is not toxic to the host to which it is administered.

The term "single package" means that the components of a kit are physically associated in or with one or more containers and considered a unit for manufacture, distribution, sale, or use. Containers include, but are not limited to, bags, boxes, bottles, shrink wrap packages, stapled or otherwise affixed components, or combinations thereof. A "single package" can also include virtual components. For instance, a kit may contain abbreviated physical instructions contained within the physical package, and instructions for accessing more detailed instructions from a virtual enviroment, such as a website for example.

The term "therapeutic" as used herein means treatment and/or prophylaxis. A therapeutic effect is obtained by avoidance, delay, suppression, remission, or eradication of a disease state associated with CMV infection.

The term "treatment" as used within the context of the present invention is meant to include therapeutic treatment as well as prophylactic, or suppressive measures for the disease or disorder. Thus, for example, the term treatment includes the administration of an agent prior to or following the onset of a disease or disorder thereby preventing or removing all signs of the disease or disorder. As another example, administration of the agent after clinical manifestation of the disease to combat the symptoms of the disease comprises "treatment" of the disease. This includes for instance, prevention of CMV propagation to uninfected cells of an organism. The phrase "diminishing CMV infection" is sometimes used herein to refer to a treatment method that involves reducing the level of infection in a patient infected with CMV, as determined by means familiar to the clinician.

Description:

Cytomegalovirus (CMV) infects multiple cell types *in vivo*, including epithelial cells, endothelial cells and fibroblasts. As summarized above in the background material, various studies have reported that the virus fuses with the plasma membranes of fibroblasts, but enters retinal pigmented epithelial cells and umbilical vein endothelial cells *via* endocytosis. Due to the relative ease of propagating CMV in cultured fibroblasts as compared with epithelial or endothelial cell cultures, studies such as the above-summarized studies have been conducted using fibroblast-propagated CMV strains. Likewise, cultured fibroblasts are typically the cell type of choice in propagating CMV for clinical applications, such as the development of attenuated virus strains for vaccines.

It has now been demonstrated in accordance with the present invention that the cell type in which CMV particles are produced has a profound influence on their behavior in subsequent rounds of infection. Thus, for example, while it was heretofore reported that that CMV enters epithelial cells by endocytosis, the present inventors have demonstrated that this is the mode of entry for CMV propagated in fibroblasts, but *not* for CMV propagated in cultured epithelial cells. Epithelial cell-propagated CMV enters epithelial cells predominantly *via* fusion with the plasma membrane. This different mode of entry has a variety of physiological consequences: it influences the kinetics with which the infection proceeds and it markedly influences the cellular response to infection. For instance virus grown in epithelial cells produces a dramatically muted cellular response as compared to cells infected with virus grown in fibroblasts. Many cellular anti-viral genes expressed after infection with fibroblast-grown virus are not expressed after infection with epithelial cell-grown virus. As a consequence, CMV grown in epithelial cells is predicted to perform differently than does a vaccine than CMV grown in fibroblasts, thus offering a new and unexpected source of diversity for the generation of CMV vaccines. Likewise, propagation of CMV in other cell types, such as endothelial cells or specialized cell types that CMV is able to infect (e.g., neurons, other cells of the central or peripheral nervous systems, smooth muscle cells, hepatocytes, stromal cells, macrophages or dendritic cells) should produce additional novel sources of diversity for the generation of CMV vaccines.

Thus, one aspect of the invention features methods of making CMV vaccines that exploit the variability associated with choosing a cell type in which to propagate the virus. Another aspect features a kit for practicing the methods described above. Another aspect of the invention features vaccine compositions for the prevention or treatment of CMV infection, and methods of immunizing an individual using such compositions. Various embodiments of these aspects of the invention are set forth below.

Methods of producing CMV vaccines:

The methods in accordance with an aspect of the invention comprise (1) providing a CMV strain or isolate; (2) propagating the strain or isolate in a cell culture of a selected cell type, and (3) harvesting CMV virions produced by growth in that cell type (referred to herein as "cell type-conditioned CMV") for use in producing a CMV vaccine.

The cell type selected for propagating the CMV prior to its use for vaccine development can be any cell line permissive for CMV infection that produces a yield of virus particles. The virus particles might be highly infectious in some assays or the particles might exhibit limited or no infectivity in many assays. Suitable cell types include, but are not limited to, (1) epithelial cell lines such as ARPE-19, which is exemplified herein and other retinal pigmented epithelial cell lines, e.g., epithelial cell line K-1034 (Ando, Y., et al. 1997, Arch. Virol.142(8): 1645-1658); HCMC, derived from normal human colonic mucosa (Smith, JD, 1986, J Virol. 60(2): 583–588); Caco-2 intestinal epithelial cells (Esclatine, A., et al., 2000, J. of Virol. 74 (1): 513-51); SW480, HCT116, HeLa, H1299, and MCF-7 (regarding the latter five, *see* Wang, D. & T. Shenk, 2005, J. Virol. 79: 10330) (2) endothelial cell lines such as HMEC-1, a human microvascular endothelial line, immortalized with SV-40 virus large T antigen (Guetta, E., et al., 2001, Cardiovascular Research 50: 538–546); HUVEC and LMVEC (regarding the latter two, *see* Wang, D. & T. Shenk, 2005, J. Virol. 79: 10330); (3) neuronal cells such as SK-N-SH, SK-N-AS and IMR-32 (*see* Wang, D. & T. Shenk, 2005, J. Virol. 79: 10330) as well as primary epithelial, endothelial, smooth muscle, macrophage and dendritic cells derived from a variety of tissue/organ sources.

Any CMV or combination of CMVs amenable to development as a vaccine is suitable for use as a source of the CMV for the method, as long as they can be grown in at least one selected cell type. In one embodiment, the CMV is human CMV (HCMV), either an isolate that has been previously isolated and characterized or a new isolate of HCMV or an HCMV-like virus. In another embodiment, the CMV originates from another primate, including but not limited to chimpanzee (Davison, AJ et al. 2003, J. Gen. Virol. 84: 17-28) and rhesus monkey (Hansen, SG et al. 2003, J. Virol. 77:6620-36; Rivailler, P et al. 2006, J. Virol. 80:4179-82). The CMV can be an unmodified virus from a selected source, or it can be a chimeric virus produced by genetic modification or combination of elements from two or more different CMV strains or isolates.

Methods of making chimeric viruses are known in the art. To this end, at least six strains of human CMV have been cloned as infectious bacterial artificial chromosomes (BAC) and sequenced (Murphy, E et al. 2003. Proc. Natl. Acad. Sci. USA 100: 14976-14981. The BAC sequences are available at GenBank Accession Nos. AC146999 (laboratory strain AD169, from which the BADrUL131 variant described herein was made); AC146851 (laboratory strain Towne); AC146904 (clinical isolate PH); AC146905 (clinical-like isolate Toledo); AC146906 (clinical isolate TR); and AC146907 (clinical isolate FIX). At least two strains of human CMV have been sequenced without prior BAC cloning, and are available at GenBank Accession Nos. BK000394 (laboratory strain AD169) and AY446894 (clinical isolate Merlin). The entire genome of a chimpanzee CMV strain is available at GenBank Accession No. AF480884. The genome sequence of two rhesus CMV strains is also available (Accession Nos. AY186194 and DQ205516). Utilizing the teachings of the present application, the skilled artisan would be able to use any of the aforementioned sequences, or any other publicly available CMV sequence to prepare chimeric CMVs or to otherwise genetically modify a CMV.

It has been demonstrated in accordance with the present invention that laboratory strains of CMV that have been passaged repeatedly in fibroblasts can be successfully conditioned by propagation on the selected cell line. For instance, as described in the Example herein, BADrUL131, a BAC clone of the repeatedly passaged AD169 HCMV strain in which the UL131 ORF has been repaired, was introduced by electroporation into cultured human foreskin fibroblasts, and the resulting virus preparation was amplified once in the epithelial cell line ARPE-19. Thus, various embodiments of the invention comprise the use of CMV (or the genomes of CMV) that has been passaged in a cell type that is different from the cell type selected for the conditioning step. For example, a CMV strain can be passaged multiple times in fibroblasts, then amplified in epithelial cells and thereafter used to produce a vaccine. It will be appreciated that the CMV can be amplified/propagated for one or more rounds in the selected cell type.

In preferred embodiments, the methods of the invention are used to produce live attenuated CMV for use as a vaccine. Methods to attenuate viruses are known in the art. Preferably, attenuated CMV exhibit a diminished capacity for infectivity, and/or pathogenicity, including latency and activation, yet remain capable of inducing an immune response that treats or protects the host against CMV infection. Examples of attenuated CMV strains include, but are not limited to, laboratory strains, such as AD169 and Towne, which replicate almost exclusively in fibroblasts. Such attenuated strains, engineered if necessary to produce the requisite surface protein or protein complexes for appropriate tropism, can be grown epithelial cells or in fibroblasts and thereafter epithelial cells as discussed above. for use in the vaccine composition of the invention.

Serial passage in cultured cells, particularly fibroblasts, can be used to attenuate CMV. Repeated passaging of virally-infected host cells is carried out *in vitro* until sufficient attenuation of the virus is achieved. Passaging may be conducted under specific environmental conditions, such as modulated temperature, pH, humidity, in order to select for viruses with reduced infectivity or pathogenicity. If this method of attenuation is used, the serially passaged virus is then amplified in the selected cell type for one or more passages to produce the CMV to be used in the vaccine compositions of the invention.

Mutagenesis can also be employed to attenuate a virus. For example, CMV virions can be exposed to ultraviolet or ionizing radiation or chemical mutagens, according to techniques known in the art. In addition to their use to produce chimeric viruses, recombinant techniques can also be used to produce attenuated CMV virions. For instance, site-directed mutagenesis, gene replacement, or gene knockout techniques can be used to derive virus strains with attenuated infectivity, pathogenicity or latency. An example of modifying a CMV by knockout mutagenesis is set forth in WO/2007/038316, which describes CMVs with genomes deleted in one or more latency-promoting genes, displaying an altered ability to enter or maintain a latent state.

In other embodiments. CMV isolated from the selected cell cultures are inactivated or killed and used in vaccine compositions. Methods of inactivating or killing viruses, e.g., with a chemical such as formalin, are well known in the art. It will be understood by the skilled artisan that the killed or inactivated CMV will comprise all or a substantial portion of the components of the viral particle, such that the diversity generated by the amplification in the selected cell type is maintained in the vaccine composition.

The methods of the invention can be used to create combinations of CMVs propagated in different selected cell types, thereby conferring an additional level of diversity to the vaccines that are produced. In one embodiment, a single CMV isolate or strain is used to infect two or more different cultured cell lines of different types, e.g., retinal epithelial cells and endothelial cells. The CMV produced by amplification in the respective cell types is then combined for use in a single vaccine. In another embodiment, two or more different clinical isolates or strains of CMV are used to infect a single selected cell line, and the multistrain or multi-isolate CMV population produced by amplification in that cell type is used to produce a vaccine. In yet another embodiment. multiple isolates or strains are used to infect two or more different cultured cell lines of different types. and the CMV populations produced by amplification in the respective cell types are combined for use in the vaccine.

Another aspect of the invention features kits for producing CMV vaccine materials in accordance with the methods described above. The kits comprise in separate containers in a single package or in separate containers in a virtual package, as appropriate for the use and kit component, aliquots of cell lines of one or more selected cell types, as well as one or more CMV isolates or strains, or vectors carrying the genomes of such CMV strains, to be introduced into and amplified in the selected cultured cell lines. Such kits also typically contain instructions, or links to instructions, for how to carry out the various steps of the method. Optionally, kits can also comprise culture medium and other reagents suitable for carrying out the cell culture and virus manipulations.

Vaccine compositions and methods of use:

Another aspect of the invention features an immunogenic composition (referred to interchangeably herein as a vaccine composition) comprising a cytomegalovirus (CMV) population or virion components thereof, admixed with a suitable pharmaceutical carrier or adjuvant, wherein the CMV is obtained *via* propagation in a selected cell type, for instance, an epithelial cell culture. As mentioned above, CMV vaccines have heretofore typically been prepared using CMV propagated in fibroblasts. However, it has been demonstrated in accordance with the present invention that propagation in epithelial cells yields virus that differs from fibroblast-propagated virus in many different ways. Virus produced in epithelial cells preferentially fuses with the plasma membrane, whereas fibroblast-derived virus mostly enters by receptor-mediated endocytosis. In addition, epithelial cell-generated virions had higher intrinsic "fusion from without" activity than fibroblast-generated particles, which influences the kinetics of infection. Furthermore, the two virus preparations trigger different cellular signaling responses, as evidenced by markedly different alterations in the transcriptional profile of infected epithelial cells.

In particular, CMV produced by propagation in epithelial cells have one or more of the following features, as compared with an equivalent strain or isolate of the virus produced by propagation in fibroblasts. First, as mentioned above, they can be distinguished by their entry into the host cells by fusion with host cell plasma membranes. CMV produced on epithelial cells also display greater virion-mediated cell-cell fusion of the host cells as compared with an equivalent CMV population isolated from cultured fibroblasts, as well as accelerated virus growth in the host cells as compared with an equivalent CMV population isolated from culture fibroblasts. In addition, they elicit a subdued cellular response as compared with equivalent CMV propagated in fibroblasts. At 10 hours post-infection about two-thirds fewer genes (~50 versus ~150 genes) exhibit a 2.5 fold or more change in expression level. In addition, epithelial-grown CMV can be characterized by the particular profile of host genes whose expression is changed (increased or decreased) following infection. These gene expression profiles are detailed in the Example, and can involve a change in expression of one or more genes represented by GenBank Accession Nos: AK094860, NM_145023, NM_133492, NM_001039580, NM_001004301, NM_001034, AI369525, AK123066, NM_005345, NM_020731, BC071797, NM_003414, NM_000800, NM_138467, AK090803, AL133118, NM_001165, BG001037, NM_024861, NM_001043, NM_016239, NM_001018084, NM_001037442, NM_017600, NM_022097, NM_175868, NM_032266, NM_003841, NM_005039, NM_145051, NM_004294, AW856073, NM_024050, AF085968, NM_080927, NM_022115, AK056703, NM_000808, NM_012377, NM_006793, NM_031466, NM_005185, NM_139173, BX360933, NM_016125, NM_002104, NM_032188, NM_004185, NM_004843 or NM_173550.

In this aspect of the invention, as in the foregoing aspects of the invention, CMV or a combination of CMVs amenable to development as a vaccine is suitable for use as a source of the aforementioned CMV population, as long as they can be grown in at least one epithelial cell line or another selected cell type. In one embodiment, the CMV is HCMV or an HCMV-like virus. In another embodiment, the CMV originates from another primate, including but not limited to chimpanzee and rhesus monkey, as described above. The CMV can be an unmodified virus from a selected source, or it can be a chimeric virus produced by genetic modification or combination of elements from two or more different CMV strains or isolates, as described above.

In preferred embodiments, the vaccine compositions comprise live attenuated CMV, which can be produced by the methods outlined above, all familiar to the skilled artisan. In other embodiments, CMV isolated from the selected cell cultures are inactivated or killed and used in vaccine compositions.

The vaccine compositions can comprise combinations of different strains or isolates of CMV, which can be propagated on a single epithelial cell cultures or on a number of different epithelial cell cultures, or on cells of another cell type, to generate additional diversity.

Furthermore. live attenuated CMV can be combined with killed or inactivated CMV. or with immunogenic components of CMV to produce a combination vaccine, e.g.. live attenuated CMV combined with heat killed CMV, or combined with material for a subunit vaccine. or a combination of all three types of materials. Examples of immunogenic CMV polypeptides and complexes suitable for subunit vaccines are described in WO 2007/146024 entitled "Cytomegalovirus Surface Protein Complex for Use in Vaccines and as a Drug Target."

The vaccine composition can further comprise one or more adjuvants. Adjuvants can be any substance that enhances the immune response to the antigens in the vaccine. Non-limiting examples of adjuvants suitable for use in the present invention include Freund's adjuvant, incomplete Freund's adjuvant. saponin, surfactants such as hexadecylamine, octadecylamine. lysolecithin, demethyldioactadecyl ammonium bromide. N,N-dioctadecyl-N'-N-bis (2-hydroxyethylpropane diamine). methoxyhexa-decyl-glycerol. pluronic polyols. polyanions such as pyran. diethylaminoethyl (DEAE) dextran. dextran sulfate. polybrene. poly IC. polyacrylic acid, carbopol. ethylene maleic acid, aluminum hydroxide, and aluminum phosphate peptides. oil or hydrocarbon emulsions, and the like.

Vaccines can be formulated in aqueous solutions such as water or alcohol, or in physiologically compatible buffers such as Hanks' solution. Ringer's solution. or physiological saline buffer. including PBS. Vaccine formulations can also be prepared as solid form preparations which are intended to be converted, shortly before use. to liquid form preparations suitable for administration to a subject. for example. by constitution with a suitable vehicle. such as sterile water, saline solution. or alcohol. before use.

The vaccine compositions can also be formulated using sustained release vehicles or depot preparations. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus. for example. the vaccines may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins. or as sparingly soluble derivatives. for example. as a sparingly soluble salt. Liposomes and emulsions can be used as delivery vehicles suitable for use with hydrophobic formulations. Sustained-release vehicles may. depending on their chemical nature. release the antigens over a range of several hours to several days to several weeks to several months.

The vaccine compositions may further include one or more antioxidants. Exemplary reducing agents include mercaptopropionyl glycine. N-acetylcysteine, β-mercaptoethylamine, glutathione, ascorbic acid and its salts. sulfite, or sodium metabisulfite, or similar species. In addition. antioxidants can also include natural antioxidants such as vitamin E. C. leutein, xanthine. beta carotene and minerals such as zinc and selenium.

Vaccine compositions may further incorporate additional substances to function as stabilizing agents. preservatives. buffers, wetting agents. emulsifying agents. dispersing agents. and monosaccharides. polysaccharides, and salts for varying the osmotic balance. The vaccines can further comprise immunostimulatory molecules to enhance vaccine efficacy. Such molecules can potentiate the immune response. can induce inflammation, and can be any lymphokine or cytokine. Nonlimiting examples of cytokines include interleukin (IL)-1, IL-2. IL-3, IL-4. IL-12, IL-13, granulocyte-macrophage colony stimulating factor (GMCSF). macrophage inflammatory factor. and the like.

Vaccines can be formulated for and administered by infusion or injection (intravenously. intraarterially. intramuscularly, intracutaneously, subcutaneously, intrathecally, intraduodenally. intraperitoneally, and the like). The vaccines can also be administered intranasally. vaginally. rectally. orally. topically. buccally. transmucosally, or transdermally.

An effective antigen dosage to treat against CMV infection can be determined empirically. by means that are well established in the art. The effective dose of the vaccine may depend on any number of variables, including without limitation, the size, height. weight. age. sex, overall health of the subject, the type of formulation, the mode or manner or administration. whether the virus is active or latent. whether the patient is suffering from secondary infections. or other related conditions.

Vaccine regimens can also be based on the above-described factors. Vaccination can occur at any time during the lifetime of the subject, including development of the fetus through adulthood. Supplemental administrations. or boosters. may be required for full protection. To determine whether adequate immune protection has been achieved, seroconversion and antibody titers can be monitored in the patient following vaccination.

The following example is provided to describe the invention in more detail. It is intended to illustrate. not to limit. the invention.

EXAMPLE

Human Cytomegalovirus Uses Two Distinct Pathways To Enter Retinal Pigmented Epithelial Cells

The experimental results described in this example demonstrate that HCMV produced in two different cell types enters epithelial cells *via* different pathways. Virions generated in epithelial cells preferentially enter *via* fusion at the plasma membrane, whereas virions from fibroblasts enter by pH-dependent endocytosis. The two virus preparations induced markedly different cellular responses.

Materials and Methods

Biological reagents. Human foreskin fibroblasts (HFFs) at passage 10 to 15 were maintained in medium with 10% newborn calf serum. Human MRC-5 embryonic lung fibroblasts and ARPE-19 retinal pigmented epithelial cells (American Type Culture Collection) at passage 24 to 34 were maintained in medium with 10% fetal bovine serum. Human renal proximal tubular epithelial cells (hRPTECs) (Cambrex) were grown in medium with 10% fetal bovine serum and used at passage 4 to 5.

BAD*wt* is derived from a BAC clone of the AD169 HCMV strain: BAD*r*UL131 (19, 21) is a derivative of BAD*wt* in which the UL131 ORF has been repaired; BFX*wt* is derived from a BAC clone of the VR1814 clinical HCMV isolate. Viruses were prepared by electroporation of BAC DNAs into HFFs, and the resulting virus preparation was amplified once in ARPE-19 cells or HFFs, unless otherwise specified. Cell-free virions were partially purified by centrifugation through a sorbitol cushion and resuspended in serum-free medium. Virus titers were determined by plaque assay on MRC-5 cells. Neutralization of BAD*r*UL131 was assayed by plaque reduction assay (19), by using purified anti-pUL130 monoclonal antibody (3E3) (19).

Anti-IE1 monoclonal antibody 1B12 was described previously (21). Rabbit anti-Sp100 polyclonal antibody (Chemicon) was used to visualize the ND10s.

Electron microscropy. ARPE-19 cells were exposed to virus at 4°C for 1 h, unbound virus was removed by two washes with cold PBS, growth medium (37°C) was added for 15 min, cells were rinsed with phosphate-buffered saline (PBS), fixed and processed for electron microscopy, and examined with an FEI Tecnai-T12 microscope at 80 kv.

Assay for the dependence of infection on endosome acidification. ARPE-19 cells were pretreated with NH_4Cl or Bafilomycin A1 (BFA) (Sigma) for 1 h at 37 °C, followed by infection in the continued presence of the inhibitor. 16 h later, cultures were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. IE1 was identified by immunofluorescence using monoclonal antibody 1B12 (21) plus Alexa 546-conjugated secondary antibody and nuclei were stained with DAPI. Inhibition was calculated as the percentage of IE1-expressing drug-treated relative to untreated cells.

Analysis of the fusion activity of virion proteins. To assay "fusion from without", ARPE-19 cells were grown to 90% confluence and infected. After 1 h at 37°C, the inoculum was removed and medium containing 200 μg/ml of phosphonoformic acid (PFA) was added to inhibit viral DNA synthesis. Fusion was monitored by visual inspection for syncytium formation.

A luciferase reporter assay was adapted to quantitatively analyze virion fusion activity. Reporter and effector ARPE-19 cells were prepared by electroporation (90-95% efficiency) with a plasmid carrying a luciferase gene under the control by a T7 promoter and a pcDNA3-T7 polymerase plasmid, respectively. At 24 h post transfection, the cells were mixed at a 1:1 ratio, and incubated at 37°C for an additional 16 h. The mixed populations were then exposed to HCMV virions at 4°C for 1 h, after which the monolayer was washed twice with cold PBS followed by addition of buffers (PBS with 10 mM 2-(N-morpholino)ethanesulfonic acid and 10 mM HEPES) with a final pH ranging of 4.5 to 8. After 3 min at 37 °C, the buffers were removed, and normal growth medium was added. At 6 hpi, the cells were lysed, and luciferase activity was assayed using a luciferase reporter assay system (Promega).

Assay of cellular transcriptional responses. Confluent ARPE-19 cells were serum starved for 24 h, followed by mock infection or infection. Total RNA was extracted at 6 or 10 hpi by using Trizol (Invitrogen), and purified with an RNeasy column (Qiagen). The RNA samples were amplified and labeled (cyanine-3) with the Agilent low RNA input fluorescent linear amplification kit. To control for chip to chip variation, a reference RNA (Clontech) was labeled (cyanine-5) and co-hybridized with the probes prepared from mock or HCMV-infected cells. The hybridization was performed in duplicate with Aligent Human 44K oligonucleotide arrays. Arrays were scanned using an Agilent scanner at 5 micron resolution, and images were analyzed with Agilent Feature Extraction software to determine the intensities of fluorescent signals for hybridized spots and for background subtraction. Agilent GeneSpring GX software was used for normalization and quantification of relative RNA changes.

Results

Fibroblast-derived virions activate immediate-early gene expression in ARPE-19 cells with slower kinetics than epithelial cell-derived virions. The AD169 HCMV strain (BAD*wt*) replicates poorly in ARPE-19 epithelial cells due to a mutation in its UL131 gene (10, 21). Repair of the mutation in AD169, producing BAD*r*UL131, restores epithelial cell tropism (21) by allowing production of a gH/gL/pUL128/pUL130/pUL131 virion glycoprotein complex that is required for successful entry into these cells (19, 20).

BAD*r*UL131 grown in ARPE-19 epithelial cells (*epi*BAD*r*UL131) initiates its program of gene expression in epithelial cells more rapidly than BAD*r*UL131 grown in HFF fibroblasts (*fibro*BAD*r*UL131) (Fig. 1A). When ARPE-19 cells were infected with *epi*BAD*r*UL131, ~17% of the cells expressed detectable IE1 protein at 6 h post infection (hpi). IE1 expression was accompanied by disruption of ND10s in the nucleus. In contrast, infection with *fibro*BAD*r*UL131 led to IE1 expression in only 2.8% of ARPE-19 cells at 6 hpi. The number of IE1-expressing cells, however, increased with time. There was no significant difference in the percentage of IE1-expressing ARPE-19 cells at 24 hpi with virus produced in the two cell types (Fig. 1B).

Virions produced in HFFs versus ARPE-19 cells enter ARPE-19 cells via distinct pathways. An electron microscopic examination of virus entry was performed to determine if the different kinetics of IE1 accumulation for ARPE-19 cell-derived virus versus HFF-derived virus resulted from an event prior to the onset of viral gene expression. ARPE-19 cells incubated with *epi*BAD*r*UL131 or *fibro*BAD*r*UL131 were permitted to attach at the cell surface at 4 °C, and cultures were shifted to 37 °C for 15 min to allow internalization before processing for microscopy. For each sample, 40-50 cells were examined, with at least 90% of the cells showing either intact virions or capsids. The number of virus particles in each cell varied from 2-8, with most cells showing 2- 3 particles.

In *epi*BAD*r*UL131-infected ARPE-19 cells, virions were found almost exclusively at the cell surface, with about 97% of the virions at the apical membrane. Some particles were close to the cells but the section did not reveal evidence of contact (Fig. 2A, panel a), and others were captured in the process of fusion at the plasma membrane (Fig. 2A, panels b and c). Capsids beneath the inner surface of the membrane were observed rarely; in fact, only two examples were identified (Fig. 2A, panels d and e). No enveloped virions were found inside the cells. This result indicates that *epi*BAD*r*UL131 enters the ARPE-19 cells by fusion with the plasma membrane. In contrast, *fibro*BAD*r*UL131-infected cells contained virions at the cell membrane (~65% of total) and inside the cell within vesicles (~35% of total) (Fig. 2B). The particles within vesicles were enveloped, indicating they entered by endocytosis.

Entry of the BFX*wt* clinical isolate propagated in fibroblasts was also examined. This clinical isolate accumulated in vesicles within ARPE-19 cells (Fig. 2C), supporting the validity of BAD*r*UL131 as a model for cell entry by a clinical isolate of HCMV.

Infection of ARPE-19 cells by fibroblast- but not epithelial cell-derived virus is pH dependent. Many viruses that enter cells by endocytosis (1, 4, 10) require acidification of endosomes for the virion envelope to fuse with the endosomal membrane and release the capsid into the cytoplasm. NH_4Cl, which buffers endosomal pH, and bafilomycin A1 (BFA), which blocks the endosomal ATPase proton pump, were tested for their effect on infection of ARPE-19 cells. After pretreatment with either agent, cells were infected and cultured in drug-containing medium for a further 16 h. Successful infections were scored by assaying for IE1-positive cells. Consistent with the ultrastructural analysis described above, pretreatment with either agent had only a modest effect on *epi*BAD*r*UL131 infection (Fig. 3A). In contrast, both agents inhibited IE1 expression after *fibro*BAD*r*UL131 infection in a dose dependent manner, indicating that the entry of fibroblast-generated virus was dependent on endosomal acidification. The fact that the agents had little effect on entry by *epi*BAD*r*UL131 shows that the inhibition of *fibro*BAD*r*UL131 did not result from toxicity.

It was next determined whether virus grown in other types of epithelial cells and fibroblasts display the same properties as ARPE19- and HFF-derived virions. Virus stocks from hRPTEC epithelial cells and MRC-5 fibroblasts were used to infect ARPE-19 cells after treatment with NH_4Cl or BFA, and they responded to the inhibitors exactly as did virus grown in ARPE-19 cells or HFFs (Fig. 3B, left panel). Thus, BAD*r*UL131 produced in two different fibroblasts was substantially more sensitive to the inhibitors than virus produced in two different epithelial cell lines.

The effect of endosomal pH on entry of the BFX*wt* clinical isolate into ARPE-19 cells was also assayed (Fig. 3B, right panel). NH_4Cl or BFA significantly reduced the number of IE1-positive ARPE-19 cells produced by infection with fibroblast-generated BFX*wt*, but only a slight inhibition was observed after infection with epithelial cell-derived BFX*wt*.

Virions produced in epithelial cells have higher intrinsic fusion activity than virions from fibroblasts. As is the case for other herpes viruses, HCMV clinical isolates promote cell-cell fusion that can be detected as early as 3-5 hpi. The rapid production of syncytia without *de novo* synthesis of virus envelope proteins indicates that it is promoted by "fusion from without", a process by which enveloped virions directly fuse target cells. Since BAD*r*UL131 produced in epithelial cells versus fibroblasts enters epithelial cells differently, the possibility that they would exhibit different "fusion from without" activities was tested.

Mock-infected ARPE-19 cells exhibited no syncytia (Fig. 4A), and syncytia were rarely found after infection with *fibro*BAD*r*UL131 (Fig. 4B). In contrast, after exposure to *epi*BAD*r*UL131, cell-cell fusion was detected as early as 6 hpi, and 20-30% of the nuclei were aggregated in syncytia by 24 hpi (Fig. 4C). Cells were treated with PFA, which blocks progression to the late phase of infection, so the fusion must have been induced by *epi*BAD*r*UL131 particles and not by newly expressed virion proteins.

A luciferase reporter assay was used to quantify the fusion activity of viral particles as well as the effects of pH on fusion from without. Reporter and effector cells received a plasmid containing a luciferase gene driven by a T7 promoter or a T7 RNA polymerase expression plasmid, respectively. The two ARPE-19 derivatives were mixed, and infection-dependent fusion was quantified by assaying luciferase expression. *epi*BAD*r*UL131 consistently induced higher fusion activity than *fibro*BAD*r*UL131 (Fig. 4D). At pH 7-8, the activity of *fibro*BAD*r*UL131 was ~3-fold lower than that of *epi*BAD*r*UL131. When the cells were treated with low pH buffers after virus adsorption, both virus preparations mediated modestly enhanced fusion. BAD*wt* did not induce fusion in this assay.

The mode of entry does not alter HCMV cell tropism. As discussed above, there is precedent for a herpesvirus to favor entering a specific cell type depending on the cell in which the infecting virus was produced. This phenomenon is different than the one that was observed as described above, i.e., HCMV preparations from different cell types enter epithelial cells by different mechanisms. Nevertheless, it remained possible that the different entry mechanisms would impact on the efficiency of replication and yield, resulting in a tropic effect. Therefore, experiments were conducted to determine whether the mode of entry influenced HCMV plaque production on epithelial cells as compared to fibroblasts (Table 1). Stocks of BAD*r*UL131 were produced in ARPE-19, hRPTEC, HFF or MRC-5 cells and assayed for plaque formation on ARPE-19 or MRC-5 cells (Table 1). Although slightly more plaques were produced on ARPE-19 than MRC-5 cells, neither epithelial cell- nor fibroblast-derived virus preferentially generated plaques on one cell type compared to the other.

TABLE 1. Titration of epithelial cell derived or fibroblast derived BAD*r*UL131 in ARPE19 and MRC5 cells ($x10^5$)

Source of replication[a]	Target cells		Ratio[b]
	ARPE-19	MRC5	
ARPE-19	8.8	3.4	2.6
hRPTEC	2.9	1.9	1.5
MRC5	4.3	2.7	1.6
HFF	6.8	2.7	2.5

[a] 2×10^5 pfu of BAD*r*UL131 originally titrated in HFFs were used to infect ARPE-19 or MRC5 cells.
[b] Ratio of ARPE-19 titer in relation to MRC5 titer.

pUL130-specific antibody blocks ARPE-19 infection by both epithelial- and fibroblast-derived virus. A pUL130-specific antibody, which neutralizes HCMV infection of epithelial cells (19), was able to block ARPE-19 infection by either mode of entry (Fig. 5A). It inhibited infection by both viruses in a dose dependent manner, although *epi*BAD*r*UL131 was somewhat more sensitive to neutralization than *fibro*BAD*r*UL131. The ability of the antibody to inhibit both modes of entry reinforces the conclusion that the pUL130-containing complex functions whether fusion occurs at the plasma membrane or the endosomal membrane.

It has been reported previously that the gH/gL/pUL128/pUL130/pUL131 complex is dispensable for HCMV to be internalized by endothelial or epithelial cells, because laboratory strains lacking this complex are efficiently endocytosed (10). However, subsequent fusion with endosomal membrane and escape into the cytoplasm requires the complex. Consistent with these earlier results, the antibody to pUL130 did not block binding or internalization of *epi*BAD*r*UL131, *fibro*BAD*r*UL131 or BAD*wt* when assayed on ARPE-19 cells (Fig 5B). However, the total amount of internalized fibroblast-derived virus was lower than that of the epithelial cell-derived virus. This might reflect a reduced rate of internalization, which would be consistent with the delay in onset of IE1 expression by the fibroblast-derived virus (Fig. 1).

***epi*BAD*r*UL131 and *fibro*BAD*r*UL131 induce different transcriptional responses in ARPE-19 cells.** Like many other viruses, HCMV modulates cellular signaling pathways during entry. One consequence of the altered intracellular signaling is a dramatic change in the cellular transcriptome, which results substantially from contact of virion glycoproteins with the host cell.

Accordingly, the impact of the two entry pathways on the transcriptional response of ARPE-19 cells was investigated. Cells were mock infected or infected with *epi*BAD*r*UL131 or *fibro*BAD*r*UL131, and total RNA was purified 6 or 10 h later. Relative RNA levels were analyzed by using microarrays, and infected-cell RNAs whose levels changed by a factor of $\geq$2.5 relative to mock-infected controls were identified (Tables 2-5). The distributions of RNAs with increased or decreased expression are depicted by Venn diagrams in Fig. 6A.

Table 2. Differentially transcribed genes from *epi*BADrUL131-infected ARP19 cells at 6 h after infection

Genbank	Fold Change	Gene Name
NM_020904	7.218	PEPP1
AK124132	5.97	LOC340286
AK074031	4.89	SLIM; FLJ34715
NM_058188	4.658	PRED54; MGC149386; MGC149387
NM_022047	4.578	IBP
NM_020436	3.172	DRRS; HSAL4; ZNF797; MGC133050; dJ1112F19.1
NM_001165	3.049	AIP1; API2; MIHC; CIAP2; HAIP1; HIAP1; MALT2; RNF49
NM_001039580	3.011	ASAP; FLJ21159
NM_000364	2.91	CMH2; TnTC; cTnT; CMPD2; MGC3889
NM_005031	2.866	PLM; MGC44983
L08436	2.825	CLP; FLJ43657; MGC19733
NM_145867	2.768	MGC33147
AL133118	2.731	AL133118
NM_001034	2.706	R2; RR2M
NM_020943	2.674	KIAA1604
BC039151	2.67	PABPC1L; FLJ42053; dJ1069P2.3
NM_031217	2.659	DKFZP434G2226
NM_003425	2.61	KOX5; ZNF13
NM_000499	2.58	AHH; AHRR; CP11; CYP1; P1-450; P450-C; P450DX
NM_182751	2.578	CNA43; PRO2249; MGC126776
NM_144620	2.572	MGC14816; DKFZp313O1122
NM_020359	0.4	PLSCR2
AF085968	0.396	AF085968
NM_053064	0.388	GNG2
NM_005039	0.38	PM; PMF; PMS; Ps 1; Ps 2; PRB1L; PRB1M
NM_152525	0.373	FLJ25351; FLJ40332
AK125975	0.365	FLJ43987
NM_175868	0.365	MAGE6; MAGE3B; MAGE-3b; MGC52297
NM_017600	0.358	DKFZp434M0331
NM_006650	0.355	CPX2; 921-L; CPX-2; MGC138492
NM_004294	0.343	RF1; MTTRF1; MGC47721
NM_006434	0.343	CAP; FLAF2; R85FL; SH3D5; SORB1
NM_031466	0.339	NIBP; T1; IBP; MGC4737; MGC4769; KIAA1882
NM_000808	0.324	MGC33793
NM_012377	0.324	OR7C3; OR19-18; CIT-HSP-87M17
NM_001018084	0.31	NM_001018084
NM_024050	0.304	DDA1; PCIA1; MGC2594
NM_005185	0.299	CLP
NM_022115	0.272	PFM15; ZNF298; C21orf83
NM_016125	0.259	LOC51136; MGC111090
NM_004843	0.256	CRL1; TCCR; WSX1; IL27R; zcytor1
NM_004334	0.242	CD157
NM_004185	0.233	WNT13; XWNT2
BX360933	0.229	SLC25A5
NM_032188	0.222	MOF; hMOF; FLJ14040
NM_173550	0.221	FLJ39267; FLJ46740; MGC50805

NM_002104	0.162	TRYP2

Microarray targets that hybridized with labeled RNA from epiBADrUL131-infected ARPE-19 cells were compared to mock-infected cells, and probe sets whose levels varied by ≥2.5 fold are listed. The Genebank designation, fold change and gene name are listed.

Table 3. Differentially transcribed genes from fibroBADrUL131-infected ARP19 cells at 6 h after infection

Genbank	Fold Change	Gene name
NM_183040	15.03	SDY; DBND; HPS7; My031; FLJ30031; MGC20210; DKFZP564K192
NM_001165	12.48	AIP1; API2; MIHC; CIAP2; HAIP1; HIAP1; MALT2; RNF49
NM_002852	11.21	TSG-14; TNFAIP5
NM_006509	7.008	I-REL
NM_139314	6.679	NL2; ARP4; FIAF; PGAR; HFARP; pp1158; ANGPTL2
NM_002982	5.977	HC11; MCAF; MCP1; MCP-1; SCYA2; GDCF-2
NM_025169	5.938	ZFP; ZNF64; ZKSCAN7; FLJ12738
NM_033066	5.144	DLG6; ALS2CR5
NM_001946	4.971	MKP3; PYST1
NM_000212	4.92	CD61; GP3A; GPIIIa
NM_001673	4.214	TS11
NM_004464	4.183	HBGF-5; Smag-82
NM_021101	4.072	CLD1; SEMP1; ILVASC
NM_006851	4.07	GLIPR; RTVP1; CRISP7
AK094860	3.913	AK094860
NM_052875	3.667	Pep8b; MGC10485
NM_005347	3.648	BIP; MIF2; GRP78; FLJ26106
NM_022842	3.592	CD318; TRASK; SIMA135
U16307	3.36	GLIPR; RTVP1; CRISP7
NM_000800	3.335	AFGF; ECGF; FGFA; ECGFA; ECGFB
NM_000800	3.306	HBGF1; GLIO703; ECGF-beta; FGF-alpha
NM_198833	3.257	PI8; CAP2
NM_002053	3.21	GBP1
NM_058179	3.161	PSA; EPIP; PSAT; MGC1460
NM_001004301	3.131	FLJ16542; FLJ34141
NM_180989	3.117	ITR
NM_000640	3.116	IL-13R; IL13BP; CD213A2
NM_002658	3.09	ATF; UPA; URK; u-PA
NM_018284	3.076	FLJ10961; DKFZp686E0974; DKFZp686L15228
NM_000201	3.022	BB2; CD54; P3.58
NM_005923	3.007	ASK1; MEKK5; MAPKKK5
NM_018836	3.001	MOT8; SHREW1; SHREW-1; RP3-426F10.1
NM_004556	2.971	IKBE
NM_022044	2.955	SDF2L1
NM_006611	2.954	Ly49; KLRA#; LY49L; Ly-49L; MGC126520; MGC126522
NM_014314	2.935	RIG-I; FLJ13599; DKFZp434J1111; DKFZp686N19181
NM_003897	2.906	DIF2; IEX1; PRG1; DIF-2; GLY96; IEX-1; IEX-1L
NM_006417	2.899	p44; MTAP44
NM_006187	2.877	p100; MGC133260
NR_002186	2.876	DKFZp58611420
NM_033036	2.872	GAL3ST2; GAL3ST-3; MGC142112; MGC142114

NM_014331	2.86	xCT; CCBR1
NM_003786	2.831	MLP2; MRP3; ABC31; MOAT-D; cMOAT2; EST90757
NM_001511	2.829	GRO1; GROa; MGSA; NAP-3; SCYB1; MGSA-a; MGSA alpha
NM_000189	2.827	HKII; HXK2; DKFZp686M1669
NM_001901	2.821	CCN2; NOV2; HCS24; IGFBP8; MGC102839
NM_031217	2.811	DKFZP434G2226
NM_002849	2.766	PTPRQ; EC-PTP; PCPTP1; PTP-SL; PTPBR7
NM_019891	2.764	ERO1LB
NM_002234	2.745	HK2; HCK1; PCN1; HPCN1; KV1.5; MGC117058; MGC117059
NM_198569	2.739	DREG; VIGR; PS1TP2
NM_020799	2.726	AMSH-FP; AMSH-LP; ALMalpha; FLJ31524; KIAA1373; etc
NM_014632	2.726	KIAA0750; MICAL2PV1; MICAL2PV2
NM_182920	2.721	FLJ42955; KIAA1312
NM_003483	2.715	BABL; LIPO; HMGIC; HMGI-C
NM_133492	2.706	ACER1; MGC138327; MGC138329
CR598364	2.633	ENST00000370238
NM_000970	2.62	TXREB1; SHUJUN-2; TAXREB107
NM_005444	2.617	RCD1; CNOT9; RCD1+
NM_194303	2.614	NM_194303
NM_015359	2.612	ZIP14; cig19; LZT-Hs4; KIAA0062
NM_016354	2.608	POAT; OATP1; OATP-E; OATP4A1; OATPRP1; SLC21A12
NM_015009	2.607	LNX3; SEMACAP3
AK124941	2.602	AK124941
NM_001548	2.602	G10P1; IFI56; ISG56; IFI-56; IFNAI1; RNM561; GARG-16
NM_145023	2.597	FLJ32762; DKFZp686N0559; RP11-479G22.1
NM_023070	2.592	FLJ34293; RP11-656D10.1
NM_001902	2.584	MGC9471
NM_004233	2.563	BL11; HB15
NM_020683	2.562	A3AR; AD026; bA552M11.5; RP11-552M11.7
NM_031938	2.56	FLJ34464; B-DIOX-II
NM_152649	2.55	FLJ34389
BC048263	2.543	LOC146909
XM_210365	2.527	LOC284288
NM_007107	2.515	TRAPG; SSR gamma
NM_002837	2.513	PTPB; HPTPB; FLJ44133; MGC59935; HPTP-BETA;
NM_172345	2.505	NM_172345
NM_002609	0.4	JTK12; PDGFR; CD140B; PDGFR1; PDGF-R-beta
NM_198353	0.4	KCTD8
NM_003558	0.394	MSS4; STM7
NM_001010911	0.392	bA418C1.3
NM_017644	0.391	DRE1; FLJ25796
NM_052892	0.388	FLJ45333; DKFZp686J19100
NM_175868	0.387	MAGE6; MAGE3B; MAGE-3b; MGC52297
NM_007282	0.38	RZF; MGC13689
NM_005185	0.38	CLP
NM_021990	0.378	GABRE
AK055156	0.375	FLJ30594; MGC120893; DKFZp761K2322
AF085968	0.375	AF085968
NM_019555	0.371	GEF3; STA3; XPLN; MGC118905; DKFZP434F2429

NM_004294	0.368	RF1; MTTRF1; MGC47721
NM_173039	0.365	AQPX1
BU943730	0.364	BU943730
NM_017600	0.364	DKFZp434M0331
NM_007282	0.36	RZF; MGC13689
AL713743	0.357	FLJ42875; MGC35434; DKFZp761G0122
NM_007314	0.347	ARG; ABLL
AK056190	0.345	WHRN; CIP98; USH2D; KIAA1526; RP11-9M16.1; DKFZP434N014
NM_000372	0.345	OCA1A; OCAIA
BC015929	0.338	RVR; BD73: HZF2: EAR-1r; Hs.37288
NM_012377	0.328	OR7C3; OR19-18: CIT-HSP-87M17
NM_138440	0.324	SLITL2
NM_001018084	0.317	NM_001018084
NM_000808	0.311	MGC33793
NM_033260	0.31	HFH1
NM_022160	0.309	DMO; MGC163307; MGC163309
BC018597	0.308	BC018597
NM_198404	0.305	bA321C24.3
NM_024050	0.303	DDA1; PCIA1: MGC2594
NM_031466	0.299	NIBP; T1; IBP; MGC4737; MGC4769; KIAA1882
NM_016831	0.287	GIG13
NM_022115	0.251	PFM15; ZNF298; C21orf83
NM_016125	0.249	LOC51136; MGC111090
NM_002104	0.242	TRYP2
NM_013261	0.236	LEM6; PGC1; PGC1A; PGC-1v; PPARGC1; PGC-1(alpha)
NM_002167	0.211	HEIR-1
NM_032188	0.204	MOF; hMOF; FLJ14040
BX360933	0.197	SLC25A5
NM_003862	0.194	ZFGF5; FGF-18
NM_173550	0.148	FLJ39267; FLJ46740; MGC50805
NM_004185	0.135	WNT13; XWNT2

Microarray targets that hybridized with labeled RNA from fibroBADrUL131-infected ARPE-19 cells were compared to mock-infected cells, and probe sets whose levels varied by ≥2.5 fold are listed. The Genebank designation, fold change, and gene name are listed.

Table 4. Differentially transcribed genes from *epi*BADrUL131-infected ARP19 cells at 10 h after infection

Genbank	Fold Change	Gene Name
AK094860	5.688	AK094860
NM_145023	4.19	FLJ32762; DKFZp686N0559; RP11-479G22.1
NM_133492	3.456	ACER1; MGC138327; MGC138329
NM_001039580	3.352	ASAP; FLJ21159
NM_001004301	2.982	FLJ16542; FLJ34141
NM_001034	2.911	R2; RR2M
AI369525	2.764	AI369525
AK123066	2.753	AK123066
NM_005345	2.729	HSP72; HSPA1; HSPA1B; HSP70-1
NM_020731	2.712	AHH; AHHR; KIAA1234
BC071797	2.631	BC071797
NM_003414	2.609	HZF2

NM_000800	2.576	AFGF; ECGF; FGFA; ECGFA; ECGFB; HBGF1; GLIO703; etc
NM_138467	2.571	C1orf171; FLJ40918
AK090803	2.557	SRrp35; FLJ14459; FLJ33484; FLJ41221; RP11-63L7.3
AL133118	2.529	AL133118
NM_001165	2.508	AIP1; API2; MIHC; CIAP2; HAIP1; HIAP1; MALT2; RNF49
BG001037	0.392	TXNRD1
NM_024861	0.388	FLJ22671; MGC150431; MGC150432
NM_001043	0.385	NET; NAT1; NET1; SLC6A5
NM_016239	0.384	DFNB3; MYO15; DKFZp686N18198
NM_001018084	0.383	NM_001018084
NM_001037442	0.381	RIPX; KIAA0871
NM_017600	0.377	DKFZp434M0331
NM_022097	0.369	LOC63928
NM_175868	0.356	MAGE6; MAGE3B; MAGE-3b; MGC52297
NM_032266	0.342	DKFZp434G118; DKFZp781D2023
NM_003841	0.342	LIT; DCR1; TRID; CD263; TRAILR3; MGC149501; MGC149502
NM_005039	0.339	PM; PMF; PMS; Ps 1; Ps 2; PRB1L; PRB1M
NM_145051	0.339	MGC4734; FLJ31197
NM_004294	0.336	RF1; MTTRF1; MGC47721
AW856073	0.335	AW856073
NM_024050	0.327	DDA1; PCIA1; MGC2594
AF085968	0.327	AF085968
NM_080927	0.318	ESDN; CLCP1
NM_022115	0.317	PFM15; ZNF298; C21orf83
AK056703	0.309	LOC219731
NM_000808	0.301	MGC33793
NM_012377	0.299	OR7C3; OR19-18; CIT-HSP-87M17
NM_006793	0.298	AOP1; MER5; AOP-1; SP-22; PRO1748; MGC24293; MGC104387
NM_031466	0.289	NIBP; T1; IBP; MGC4737; MGC4769; KIAA1882
NM_005185	0.286	CLP
NM_139173	0.286	MGC131641
BX360933	0.28	SLC25A5
NM_016125	0.269	LOC51136; MGC111090
NM_002104	0.251	TRYP2
NM_032188	0.248	MOF; hMOF; FLJ14040
NM_004185	0.245	WNT13; XWNT2
NM_004843	0.237	CRL1; TCCR; WSX1; IL27R; zcytor1
NM_173550	0.208	FLJ39267; FLJ46740; MGC50805

Microarray targets that hybridized with labeled RNA from epiBADrUL131-infected ARPE-19 cells were compared to mock-infected cells, and probe sets whose levels varied by ≥2.5 fold are listed. The Genebank designation, fold change and gene name are listed.

Table 5. Differentially transcribed genes from *fibro*BADrUL131-infected ARP19 cells at 10 h after infection

Genbank	Fold Change	Gene Name
AK094860	11.26	AK094860
NM_033066	8.751	DLG6; ALS2CR5
NM_145023	6.529	FLJ32762; DKFZp686N0559; RP11-479G22.1
NM_152377	4.463	FLJ44073; MGC34837
NM_002310	4.386	SWS; SJS2; STWS; CD118
NR_001279	4.051	LOC164380; MGC26611; MGC26924

NM_005345	4.008	HSP72; HSPA1; HSPA1B; HSP70-1
NM_006417	3.879	p44; MTAP44
NM_017638	3.783	p28b; FLJ20045
NM_001165	3.695	AIP1; API2; MIHC; CIAP2; HAIP1; HIAP1; MALT2; RNF49
NM_000640	3.659	IL-13R; IL13BP; CD213A2
NM_001673	3.313	TS11
NM_002526	3.274	NT; eN; NT5; NTE; cNT; CD73; E5NT
NM_003786	3.244	MLP2; MRP3; ABC31; MOAT-D; cMOAT2; EST90757
NM_005527	3.229	hum70t; HSP70-HOM
NM_018372	3.224	RIF1; FLJ11269; RP11-96K19.1
NM_133492	3.16	ACER1; MGC138327; MGC138329
NM_033160	3.101	FLJ32813; MGC35232; DKFZp572C163
DB318210	3.094	DB318210
NM_182751	3.093	CNA43; PRO2249; MGC126776
NM_180989	3.089	ITR
NM_005345	3.048	HSP72; HSPA1; HSPA1B; HSP70-1
NM_005345	3.029	HSP72; HSPA1; HSPA1B; HSP70-1
NM_000212	2.993	CD61; GP3A; GPIIIa
NM_145867	2.991	MGC33147
NM_021813	2.976	BACH2
NM_006187	2.943	p100; MGC133260
CR594200	2.942	LOC643837
NM_012419	2.941	RGSZ2; RGS-17; hRGS17
AF038194	2.923	AF038194
NM_018664	2.921	SNFT; BATF3; JUNDM1
NM_017577	2.907	FLJ35862; FLJ40464
NM_144633	2.887	ELK; ELK1; elk3; Kv12.1
NM_144620	2.86	MGC14816; DKFZp313O1122
NM_001004301	2.859	FLJ16542; FLJ34141
NM_002852	2.847	TSG-14; TNFAIP5
NM_007107	2.839	TRAPG; SSR gamma
NM_032778	2.836	MDIG; NO52; MINA53; FLJ14393; DKFZp762O1912
NM_032523	2.828	ORP6; FLJ36583; MGC59642
NM_005515	2.808	HB9; SCRA1; HOXHB9
NM_002201	2.804	CD25; HEM45
NM_152649	2.799	FLJ34389
NM_033036	2.794	GAL3ST2; GAL3ST-3; MGC142112; MGC142114
NM_006509	2.791	I-REL
NM_004233	2.789	BL11; HB15
NM_180989	2.772	ITR
NM_020988	2.735	GNAO; G-ALPHA-o; DKFZp686O0962
U16307	2.687	GLIPR; RTVP1; CRISP7
NM_003706	2.672	CPLA2-gamma; DKFZp586C0423
NM_153689	2.662	FLJ38973
NM_000800	2.653	AFGF; ECGF; FGFA; ECGFA; ECGFB; HBGF1; GLIO703; etc
BC043212	2.643	LOC402125
NM_002670	2.619	I-PLASTIN
NM_152408	2.614	FLJ35779; MGC120442; MGC120443; MGC120444
NM_198951	2.613	TG2; TGC
NM_012329	2.595	MMA; PAQR11
NM_001009954	2.589	FLJ20105; MGC131695

NM_032228	2.585	FAR1; FLJ22728; FLJ33561
AI369525	2.584	AI369525
NM_004170	2.583	EAAC1; EAAT3
NM_002930	2.571	RIN; RIBA; ROC2
AK023856	2.569	LOC339803
NM_024525	2.558	FLJ22584
NM_152649	2.553	FLJ34389
NM_181795	2.552	PRKACN2; FLJ23817
BC039151	2.549	PABPC1L; FLJ42053; dJ1069P2.3
NM_006547	2.525	IMP3; KOC1; IMP-3; VICKZ3; DKFZp686F1078
NM_000641	2.521	AGIF; IL-11
NM_145306	2.506	C10orf35
AK021804	0.398	AK021804
NM_007211	0.397	HoJ-1; C12orf2
NM_203434	0.397	MGC70833; bA247A12.2
NM_000362	0.397	SFD; K222; K222TA2; HSMRK222
AK056703	0.395	LOC219731
NM_003558	0.395	MSS4; STM7
NM_016831	0.394	GIG13
NM_024861	0.394	FLJ22671; MGC150431; MGC150432
BF514513	0.393	BF514513
NR_002819	0.392	MALAT-1
NM_002609	0.391	JTK12; PDGFR; CD140B; PDGFR1; PDGF-R-beta
NM_018027	0.391	FRMD4; FLJ10210; KIAA1294; bA295P9.4
NM_001010911	0.39	bA418C1.3
AW444553	0.389	FAM84B
AK056190	0.388	WHRN; CIP98; USH2D; KIAA1526; RP11-9M16.1
NM_175868	0.385	MAGE6; MAGE3B; MAGE-3b; MGC52297
AB051431	0.385	KIAA1644; MGC125851; MGC125852
NM_001003683	0.384	HCAM1; HSPDE1A; MGC26303
NM_004294	0.384	RF1; MTTRF1; MGC47721
NM_006516	0.383	GLUT; GLUT1; MGC141895; MGC141896
BX104999	0.382	BX104999
AL713743	0.381	FLJ42875; MGC35434; DKFZp761G0122
NM_000322	0.381	RDS; RP7; rd2; AVMD; PRPH; AOFMD; TSPAN22
NM_007314	0.381	ARG; ABLL
NM_018371	0.376	ChGn; FLJ11264; beta4GalNAcT
NR_002802	0.376	TncRNA
DB527271	0.376	DB527271
NM_006393	0.374	LNEBL; bA56H7.1; MGC119746; MGC119747
NM_013989	0.372	D2; 5DII; SelY; TXDI2
NM_017600	0.37	DKFZp434M0331
BC011595	0.369	NMB; HGFIN
AF085968	0.366	AF085968
BC018597	0.365	BC018597
NM_014729	0.362	TOX1; KIAA0808
NM_001003940	0.362	FLJ00065
NM_000372	0.358	OCA1A; OCAIA
NM_019555	0.358	GEF3; STA3; XPLN; MGC118905; DKFZP434F2429
NM_022115	0.357	PFM15; ZNF298; C21orf83
NM_198353	0.355	KCTD8

NM_032434	0.355	KIAA1805; MGC111046
AK055386	0.355	AK055386
NM_006933	0.352	SMIT; SMIT2
CR622110	0.35	CR622110
AW856073	0.347	AW856073
NM_015074	0.345	KLP; CMT2; CMT2A; CMT2A1; HMSNII
NM_032866	0.342	JACOP; FLJ14957; KIAA1749; MGC138254
NM_012377	0.342	OR7C3; OR19-18; CIT-HSP-87M17
NM_005261	0.341	KIR; MGC26294
AK023391	0.339	AK023391
NM_002214	0.339	ITGB8
NM_182728	0.339	LAT2; LPI-PC1
NM_024050	0.338	DDA1; PCIA1; MGC2594
NM_005185	0.338	CLP
NM_016613	0.337	AD021; AD036; FLJ38155; DKFZp434L142
NM_000782	0.336	CP24; CYP24; MGC126273; MGC126274; P450-CC24
NM_001624	0.335	ST4
NM_007282	0.333	RZF; MGC13689
NM_001037442	0.327	RIPX; KIAA0871
NM_004318	0.32	BAH; HAAH; JCTN; junctin; CASQ2BP1
BU943730	0.32	BU943730
NM_205849	0.319	FLJ40182
NM_000808	0.315	MGC33793
NM_033260	0.313	HFH1
NM_000916	0.309	OT-R
NM_032188	0.309	MOF; hMOF; FLJ14040
BX360933	0.306	SLC25A5
NM_014351	0.304	NST; BRSTL1; SULTX3; BR-STL-1; MGC40032; DJ388M5.3; etc
NM_002167	0.3	HEIR-1
NM_001033086	0.298	dJ631M13.5; RP11-189J1.1
NM_000372	0.292	OCA1A; OCAIA
NM_001002926	0.289	TWISTNB
AK094143	0.288	C14orf78; KIAA2019
NM_004466	0.287	GPC5
NM_031466	0.276	NIBP; T1; IBP; MGC4737; MGC4769; KIAA1882
NM_013261	0.271	LEM6; PGC1; PGC1A; PGC-1v; PPARGC1; PGC-1(alpha)
NM_000693	0.267	ALDH6; RALDH3; ALDH1A6
NM_016125	0.26	LOC51136; MGC111090
AK124390	0.23	AK124390
NM_002104	0.228	TRYP2
NM_005341	0.209	HKR3; pp9964
NM_173082	0.202	FLJ27258; FLJ37625; FLJ45012
NM_173550	0.191	FLJ39267; FLJ46740; MGC50805
NM_004185	0.133	WNT13; XWNT2
NM_021727	0.0415	CYB5RP; LLCDL3

Microarray targets that hybridized with labeled RNA from fibroBADrUL131-infected ARPE-19 cells were compared to mock-infected cells, and probe sets whose levels varied by ≥2.5 fold are listed. The Genebank designation, fold change and gene name are listed.

At 6 h after *epi*BAD*r*UL131 infection, the levels of 47 RNAs were changed as compared to mock-infected cells, and 121 RNAs were altered in *fibro*BAD*r*UL131-infected versus mock-infected cells. The set of modulated RNAs was substantially different for the two viruses; only 19 RNAs were altered after infection with either *epi*BAD*r*UL131 or *fibro*BAD*r*UL131. Although there might be several instances in which a gene was altered by one virus by a factor of ≥2.5-fold, while the other virus induced a more modest alteration that fell below the cut-off, inspection of the data revealed that this was not common. At 10 hpi, the number of host cell RNAs modulated by *epi*BAD*r*UL131 increased only slightly (50 RNAs), whereas a more substantial increase was observed for *fibro*BAD*r*UL131 (153 RNAs). At the later time, the number of RNAs modulated by both viruses increased to a limited extent (28 RNAs). The microarray results were confirmed by real time RT-PCR for one RNA that was not altered and six RNAs that were altered by infection (Fig. 6B).

To further compare the modulation of RNA levels by *fibro*BAD*r*UL131 versus *epi*BAD*r*UL131, the array results were filtered using a gene list comprised of four Gene Ontology groups: host-pathogen interaction (GO:0030383), cell communication (GO:0007154), viral life cycle (GO:0016032) and cell-cell signaling (GO:0007267). Nearly one third of the mRNAs (70 of 222) that were regulated greater than 2.5 fold in *fibro*BAD*r*UL131-infected ARPE-19 cells were present in the combined grouping (Table 6). In marked contrast, only one of 86 RNAs induced by *epi*BAD*r*UL131 was found in these four Gene Ontology groups. The two virus preparations generated substantially different transcriptional responses upon infection of epithelial cells.

Table 6. fibroBADrUL131-modified cellular RNA levels

Genbank	Fold Change 6hpi	Fold Change 10hpi	Gene Name
NM_006509	7.008	2.791	I-REL
NM_139314	6.679	2.067	NL2; ARP4; FIAF; PGAR; HFARP; pp1158; ANGPTL2
NM_002982	5.977	nc	HC11; MCAF; MCP1; MCP-1; SCYA2; GDCF-2; etc
NM_000212	4.92	2.993	CD61; GP3A; GPIIIa
NM_002310	nc	4.386	SWS; SJS2; STWS; CD118
NM_004464	4.183	2.264	HBGF-5; Smag-82
NM_021101	4.072	nc	CLD1; SEMP1; ILVASC
NM_006851	4.07	2.364	GLIPR; RTVP1; CRISP7
NM_005347	3.648	nc	BIP; MIF2; GRP78; FLJ26106
U16307	3.36	2.687	GLIPR; RTVP1; CRISP7
NM_000800	3.335	2.115	AFGF; ECGF; FGFA; ECGFA; ECGFB; HBGF1; etc
NM_002526	nc	3.274	NT; eN; NT5; NTE; eNT; CD73; E5NT
NM_005527	nc	3.229	hum70t; HSP70-HOM
NM_002053	3.21	nc	GBP1
NM_180989	3.117	nc	ITR
NM_000640	3.116	3.659	IL-13R; IL13BP; CD213A2
NM_002658	3.09	nc	ATF; UPA; URK; u-PA

NM_180989	nc	3.089	ITR
NM_018284	3.076	2.185	FLJ10961; DKFZp686E0974; DKFZp686L15228
NM_005345	nc	3.048	HSP72; HSPA1; HSPA1B; HSP70-1
NM_000201	3.022	nc	BB2; CD54; P3.58
NM_004556	2.971	nc	IKBE
NM_006611	2.954	2.263	Ly49; KLRA#; LY49L; Ly-49L; MGC126520; etc
NM_014314	2.935	2.071	RIG-I; FLJ13599; DKFZp434J1111; DKFZp686N19181
NM_003897	2.906	nc	DIF2; IEX1; PRG1; DIF-2; GLY96; IEX-1; IEX-1L
NM_006417	2.899	3.879	p44; MTAP44
NM_144633	nc	2.887	ELK; ELK1; elk3; Kv12.1
NM_006187	2.877	2.943	p100; MGC133260
NM_032778	nc	2.836	MDIG; NO52; MINA53; FLJ14393; DKFZp762O1912
NM_003786	2.831	3.244	MLP2; MRP3; ABC31; MOAT-D; cMOAT2; EST90757
NM_001511	2.829	nc	GRO1; GROa; MGSA; NAP-3; SCYB1; MGSA-a; etc
NM_001901	2.821	nc	CCN2; NOV2; HCS24; IGFBP8; MGC102839
NM_002849	2.766	nc	PTPRQ; EC-PTP; PCPTP1; PTP-SL; PTPBR7
NM_002234	2.745	nc	HK2; HCK1; PCN1; HPCN1; KV1.5; MGC117058; etc
NM_198569	2.739	2.182	DREG; VIGR; PS1TP2
NM_000970	2.62	nc	TXREB1; SHUJUN-2; TAXREB107
NM_198951	nc	2.613	TG2; TGC
NM_015359	2.612	nc	ZIP14; cig19; LZT-Hs4; KIAA0062
NM_001548	2.602	nc	G10P1; IFI56; ISG56; IFI-56; IFNAI1; RNM561; etc
NM_012329	nc	2.595	MMA; PAQR11
NM_002930	nc	2.571	RIN; RIBA; ROC2
NM_004233	2.563	2.789	BL11; HB15
NM_020683	2.562	nc	A3AR; AD026; bA552M11.5; RP11-552M11.7
NM_181795	nc	2.552	PRKACN2; FLJ23817
NM_006547	nc	2.525	IMP3; KOC1; IMP-3; VICKZ3; DKFZp686F1078
NM_000641	nc	2.521	AGIF; IL-11
NM_172345	2.505	nc	NM_172345
NM_012419	2.359	2.941	RGSZ2; RGS-17; hRGS17
NM_020988	2.188	2.735	GNAO; G-ALPHA-o; DKFZp686O0962
NM_002201	2.028	2.804	CD25; HEM45
NM_004318	0.495	0.32	BAH; HAAH; JCTN; junctin; CASQ2BP1
NM_013989	0.492	0.372	D2; 5DII; SelY; TXDI2
NM_005261	0.468	0.341	KIR; MGC26294
NM_000916	0.434	0.309	OT-R
NM_014351	0.408	0.304	NST; BRSTL1; SULTX3; BR-STL-1; MGC40032; etc
NM_002609	0.4	0.391	JTK12; PDGFR; CD140B; PDGFR1; PDGF-R-beta
NM_007211	nc	0.397	HoJ-1; C12orf2
NM_001003683	nc	0.384	HCAM1; HSPDE1A; MGC26303
NM_006516	nc	0.383	GLUT; GLUT1; MGC141895; MGC141896
NM_000322	nc	0.381	RDS; RP7; rd2; AVMD; PRPH; AOFMD; TSPAN22
NM_021990	0.378	0.49	GABRE
NM_018371	nc	0.376	ChGn; FLJ11264; beta4GalNAcT
NM_019555	0.371	0.358	GEF3; STA3; XPLN; MGC118905; DKFZP434F2429
NM_007314	0.347	0.381	ARG; ABLL
NM_002214	nc	0.339	ITGB8
NM_182728	nc	0.339	LAT2; LPI-PC1
BC015929	0.338	0.453	RVR; BD73; HZF2; EAR-1r; Hs.37288

NM_016831	0.287	0.394	GIG13
NM_013261	0.236	0.271	LEM6; PGC1; PGC1A; PGC-1v; PPARGC1; etc
NM_003862	0.194	nc	ZFGF5; FGF-18

Four GO groups were combined: host-pathogen interaction (GO:0030383), cell communication (GO:0007154), viral life cycle (GO:0016032) and cell-cell signaling (GO:0007267). The set of 9276 genes was used to filter array results from fibroBADrUL131-infected ARPE-19 cells. Genbank identifiers and gene names are shown along with the fold induction or repression at 6 and 10 hpi. Probe sets that did not change by ≥2.5 compared to mock-infected cells are designated by "nc" for no change.

Discussion

ARPE-19 epithelial cells can be infected by HCMV through two different routes: fusion at the plasma membrane or endocytosis followed by fusion at the endosomal membrane. Both modes of entry initiate a productive infection. The route of entry depends on the cell type in which the virus was propagated. HCMV from epithelial cells enters by the former route, and virus grown in fibroblasts follows the latter path. This conclusion follows from ultrastructural analysis and differential sensitivity of infection to agents that block acidification of endosomes. The observation that virus grown in epithelial cells has greater "fusion from without" activity than does virus produced in fibroblasts reinforces the view that the two virus preparations interact with ARPE-19 cells in a fundamentally different manner. Importantly, both modes of entry require pUL130 function because pUL130 antibody neutralized infection by virus produced from either source. The gH/gL/pUL128/pUL130/pUL131 complex functions at the ARPE-19 plasma membrane if the infecting virus has been produced in epithelial cells and at the endosomal membrane if the virus was grown in fibroblasts. Neutralized virus in the endosome fails to escape and presumably suffers the same fate as AD169, which lacks the pUL130-containing complex and accumulates in epithelial cell endosomes without initiating a productive infection (10).

Virus grown in fibroblasts induces IE1 protein accumulation in ARPE-19 cells after a delay relative to virus from epithelial cells, suggesting that some aspect of entry by endocytosis proceeds more slowly than entry by fusion at the plasma membrane. Many virions are evident in endosomes, but no capsids were seen in the cytoplasm after entry of fibroblast-generated virus; and capsids were found rarely in the cytoplasm of cells infected with epithelial cell-produced virus. Apparently, virions linger for a time in endosomes, but once a capsid is freed of its envelope and reaches the cytoplasm, it is rapidly disassembled.

How are HCMV virions produced in the two cell types different? It appears different "fusion from without" activities provide an indication. Not only did *epi*BAD*r*UL131 induce fusion more efficiently than *fibro*BAD*r*UL131, but lowered pH enhanced the activities of both virus preparations. Without intending to be bound or limited by any explanation of mechanism, it is possible that fusion of membranes requires a threshold of fusion activity. The ability of pUL130 antibody to neutralize both virus preparations indicates that both depend on the gH/gL/pUL128/pUL130/pUL131 complex for fusion, so experiments were devised to the hypothesis that the viruses contain different amounts of the complex. Several of its constituents were assayed, and it was found that a slightly higher ratio (~2-fold) of gH /gL/pUL128/pUL130/pUL131 to gH/gL/gO were present in *epi*BAD*r*UL131 particles than in *fibro*BAD*r*UL131 particles. The levels of gB, pp28 and pp65 were similar in the two virion preparations.

There is precedent in EBV for production of viruses with different relative amounts of a gH complex; particles produced by B cells are deficient for gH/gL/gp42 (18). However, other factors may be involved. Perhaps a constituent of the complex that was not assayed is altered. Alternatively, the ratio of the gH complex to one or more additional virion glycoprotein complexes might modify fusion activity. Finally, it may be that an unidentified cell protein, supplied to the virions when they are produced within epithelial cells or fibroblasts, might alter the complex.

Are there physiological consequences to the two modes of entry? *epi*BAD*r*UL131 and *fibro*BAD*r*UL131 induced markedly different cellular transcriptional responses after infection of ARPE-19 cells. Assuming that the difference is indeed due to virions or virions plus specifically associated cellular factors, the microarray experiment demonstrates a strikingly different transcriptional response to infection. Endocytosis is intimately involved in the regulation of signaling by cell surface molecules. As a consequence, a virus might modulate cell signaling, and the cellular transcriptome, differently if it enters by fusion at the plasma membrane versus endocytosis. The differences in cell signaling likely have physiological consequences that are not detected in cultured cells, such as effects on virus spread, immune evasion, or virulence.

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The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the appended claims.

What is Claimed:

1. A method of making a cytomegalovirus (CMV) vaccine comprising:

    a) passaging a strain or an isolate of CMV in cultured cells of a first selected cell type;

    b) amplifying the strain or isolate of CMV in a second selected cell type, thereby producing a cell type-conditioned CMV, wherein the first and second cell types are different; and

    b) producing a CMV vaccine from the cell type-conditioned CMV.

2. The method of claim 1, wherein the strain or isolate of CMV is a human CMV strain or isolate.

3. The method of claim 1, wherein the first and second selected cell types are selected from the group consisting of epithelial cells, endothelial cells, fibroblasts, neuronal cells, smooth muscle cells, macrophages, dendritic cells and stromal cells.

4. The method of claim 3, wherein the first selected cell type is a fibroblast and the second selected cell type is an epithelial cell.

5. The method of claim 1, comprising producing the cell type-conditioned CMV in two or more different second selected cell types and combining the cell type-conditioned CMV to produce the CMV vaccine.

6. The method of claim 1, further comprising passaging and amplifying two or more CMV strains or isolates, and combining the cell type-conditioned CMV to produce the CMV vaccine.

7. The method of claim 1, comprising producing a live attenuated CMV vaccine.

8. The method of claim 1, comprising producing an inactivated or killed CMV vaccine.

9. A CMV vaccine produced by the method of any one of claims 1-8.

10. A vaccine composition comprising a cytomegalovirus (CMV) population or virion components thereof, admixed with a suitable pharmaceutical carrier or adjuvant, wherein the CMV population is isolated from an epithelial cell culture.

11. The vaccine composition of claim 10, wherein the CMV is human CMV.

12. The vaccine composition of claim 10, wherein the CMV population isolated from the epithelial cell culture is characterized by one or more features in subsequently infected host cells comprising:

a) entry into the host cells by fusion with host cell plasma membranes:

b) greater virion-mediated cell-cell fusion of the host cells as compared with an equivalent CMV population isolated from cultured fibroblasts:

c) accelerated virus growth in the host cells as compared with an equivalent CMV population isolated from culture fibroblasts:

d) elicitation of a cellular response involving changes in expression greater than or equal to 2.5 fold of about two thirds fewer genes than a response elicited by an equivalent CMV population isolated from culture fibroblasts at 10 hours post-infection: or

e) elicitation of a cellular response involving a change in expression of one or more genes represented by GenBank Accession Nos: AK094860, NM_145023, NM_133492, NM_001039580, NM_001004301, NM_001034, AI369525, AK123066, NM_005345, NM_020731, BC071797, NM_003414, NM_000800, NM_138467, AK090803, AL133118, NM_001165, BG001037, NM_024861, NM_001043, NM_016239, NM_001018084, NM_001037442, NM_017600, NM_022097, NM_175868, NM_032266, NM_003841, NM_005039, NM_145051,

NM_004294, AW856073, NM_024050, AF085968, NM_080927, NM_022115, AK056703, NM_000808, NM_012377, NM_006793, NM_031466, NM_005185, NM_139173, BX360933, NM_016125, NM_002104, NM_032188, NM_004185, NM_004843 or NM_173550.

13. The vaccine composition of claim 10, further comprising a CMV population or virion components thereof isolated from a cell culture of another cell type.

14. The vaccine composition of claim 13, wherein the other cell type is a fibroblast cell type.

15. The vaccine composition of claim 10, wherein the CMV population comprises two or more CMV strains or clinical isolates grown in epithelial cell cultures.

16. The vaccine composition of claim 10, comprising a live attenuated CMV vaccine.

17. The vaccine composition of claim 10, comprising an inactivated CMV vaccine.

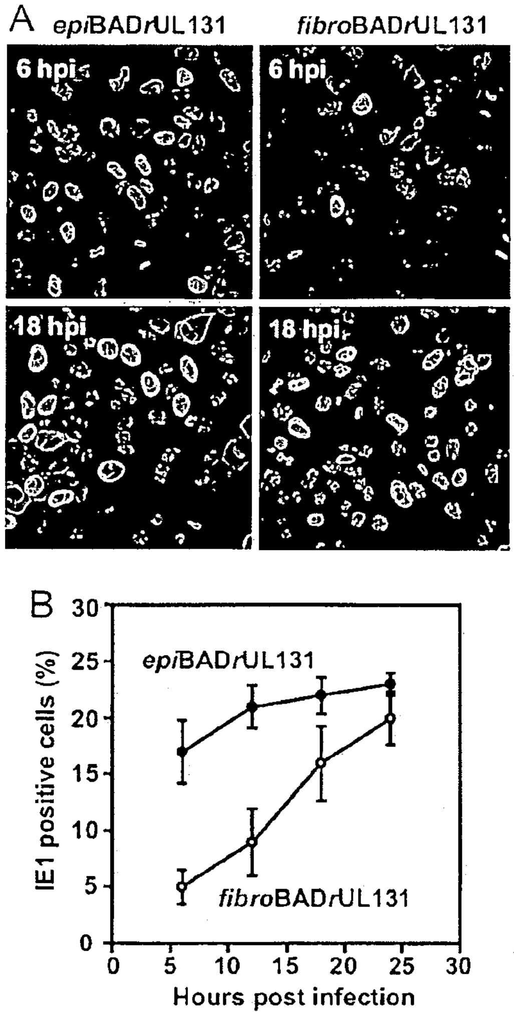
A
*epi*BAD*r*UL131
*fibro*BAD*r*UL131
6 hpi
6 hpi
18 hpi
18 hpi
B
IE1 positive cells (%)
*epi*BAD*r*UL131
*fibro*BAD*r*UL131
Hours post infection
0
5
10
15
20
25
30

Fig. 1

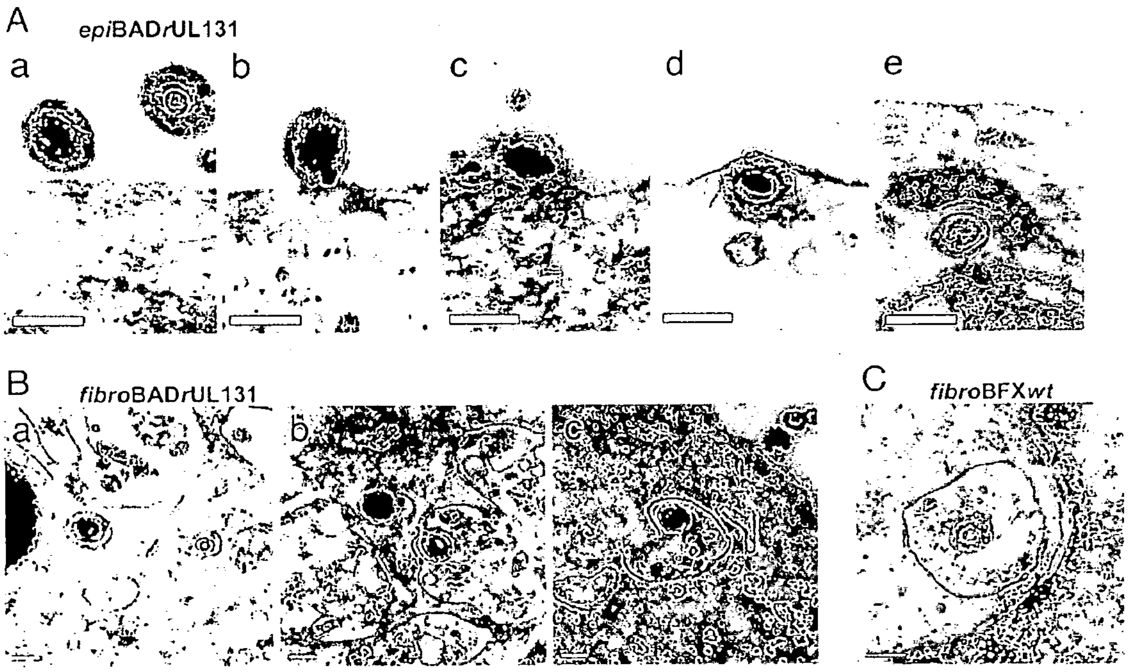

Fig. 2

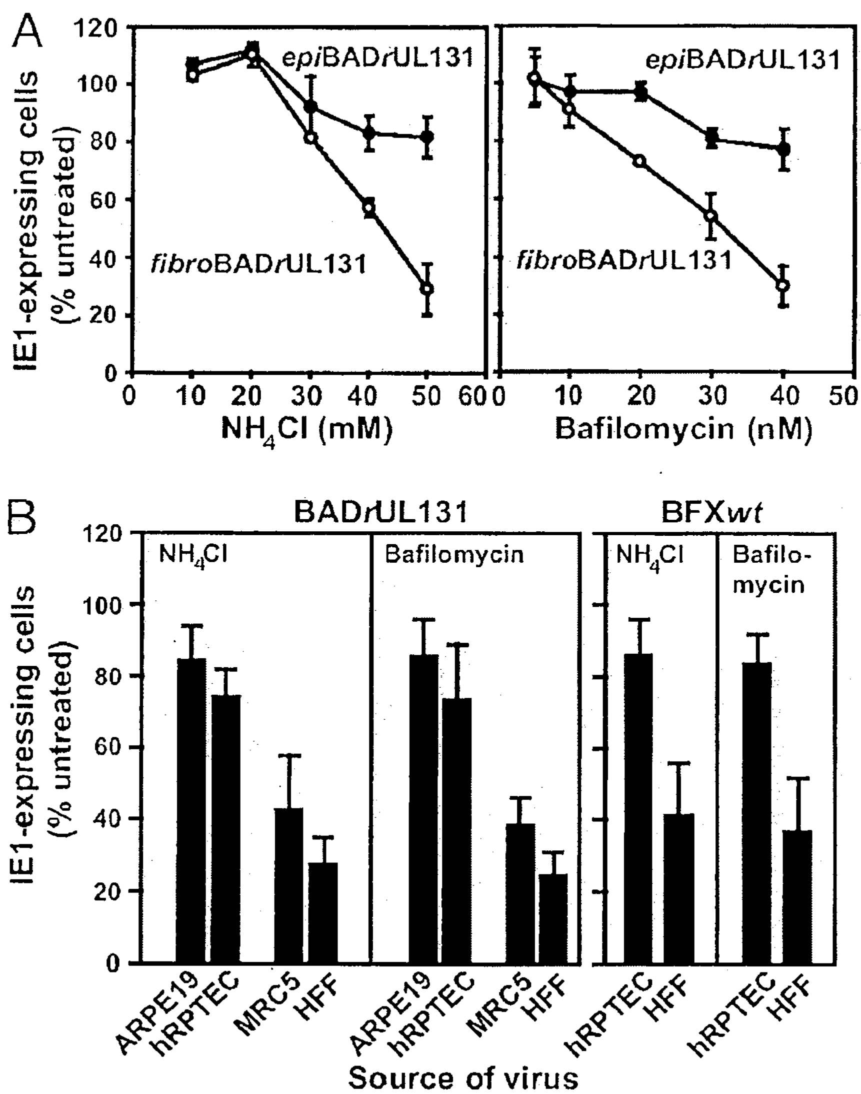
A
IE1-expressing cells
(% untreated)
120
100
80
60
40
20
0
epiBADrUL131
fibroBADrUL131
0 10 20 30 40 50 60
NH4Cl (mM)
0 10 20 30 40 50
Bafilomycin (nM)
B
BADrUL131
BFXwt
NH4Cl
Bafilomycin
NH4Cl
Bafilo-
mycin
ARPE19
hRPTEC
MRC5
HFF
hRPTEC
HFF
Source of virus

Fig. 3

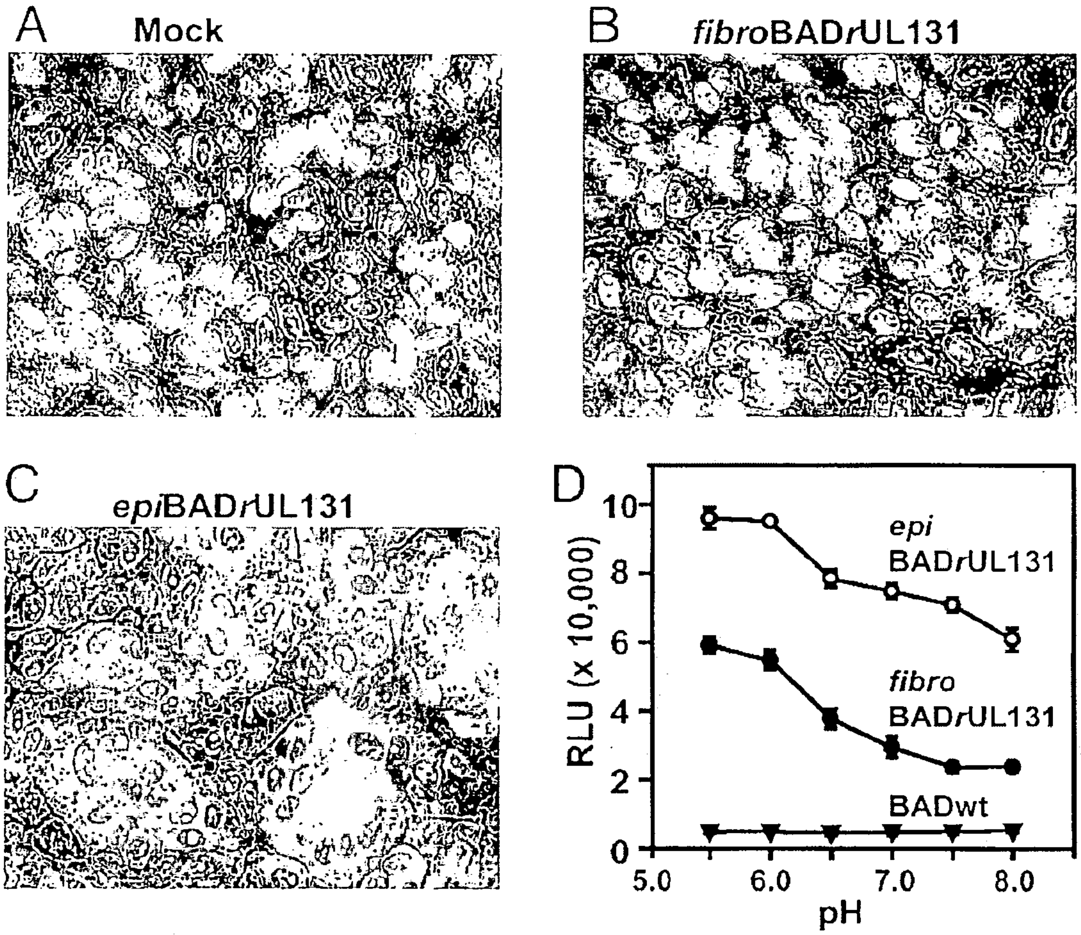
A
Mock
B
fibroBADrUL131
C
epiBADrUL131
D
epi
BADrUL131
fibro
BADrUL131
BADwt
RLU (x 10,000)
pH
0
2
4
6
8
10
5.0
6.0
7.0
8.0

Fig. 4

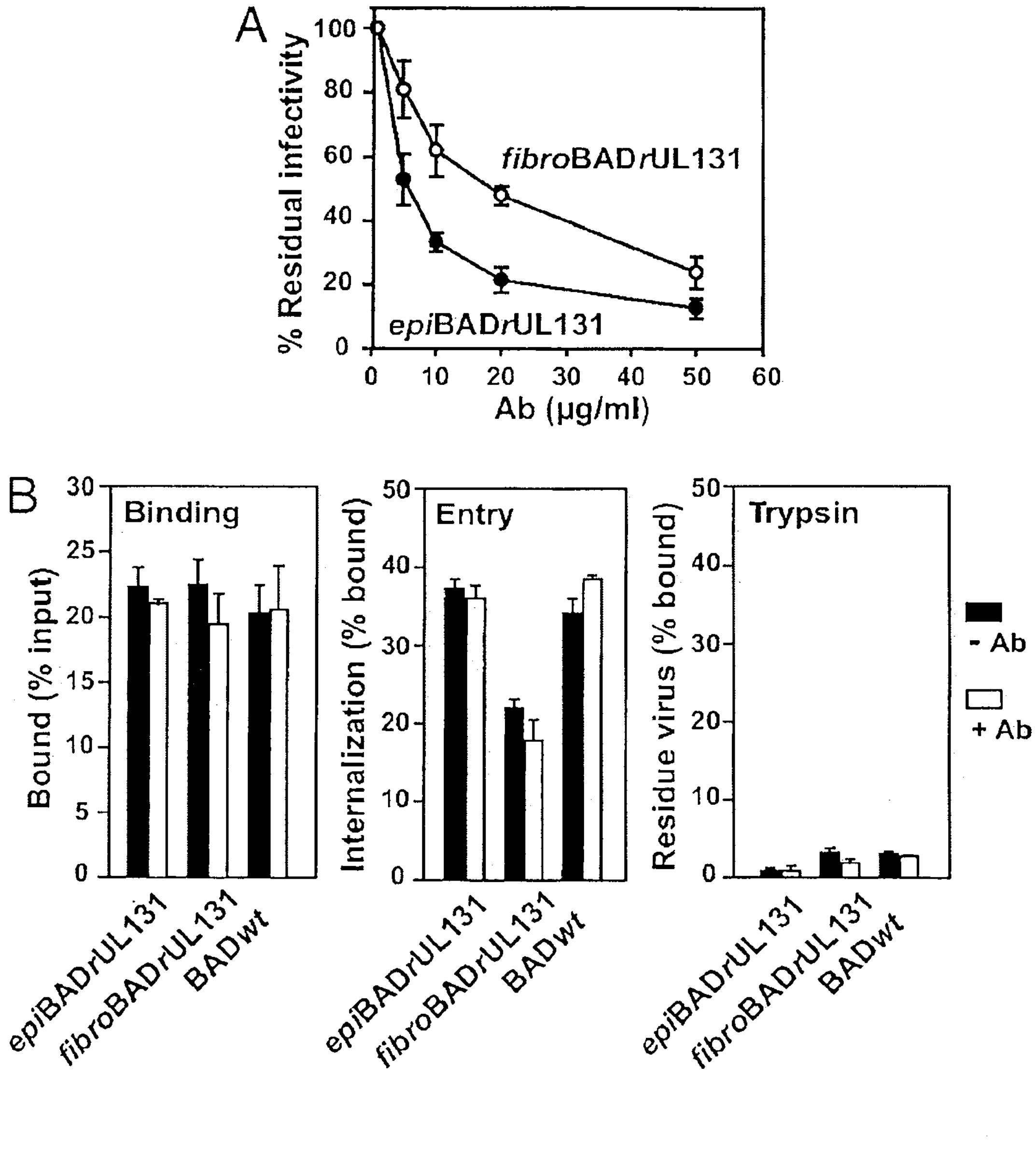
A
% Residual infectivity
100
80
60
40
20
0
fibroBADrUL131
epiBADrUL131
0
10
20
30
40
50
60
Ab (µg/ml)
B
Binding
Bound (% input)
30
25
20
15
10
5
0
epiBADrUL131
fibroBADrUL131
BADwt
Entry
Internalization (% bound)
50
40
30
20
10
0
Trypsin
Residue virus (% bound)
- Ab
+ Ab

Fig. 5

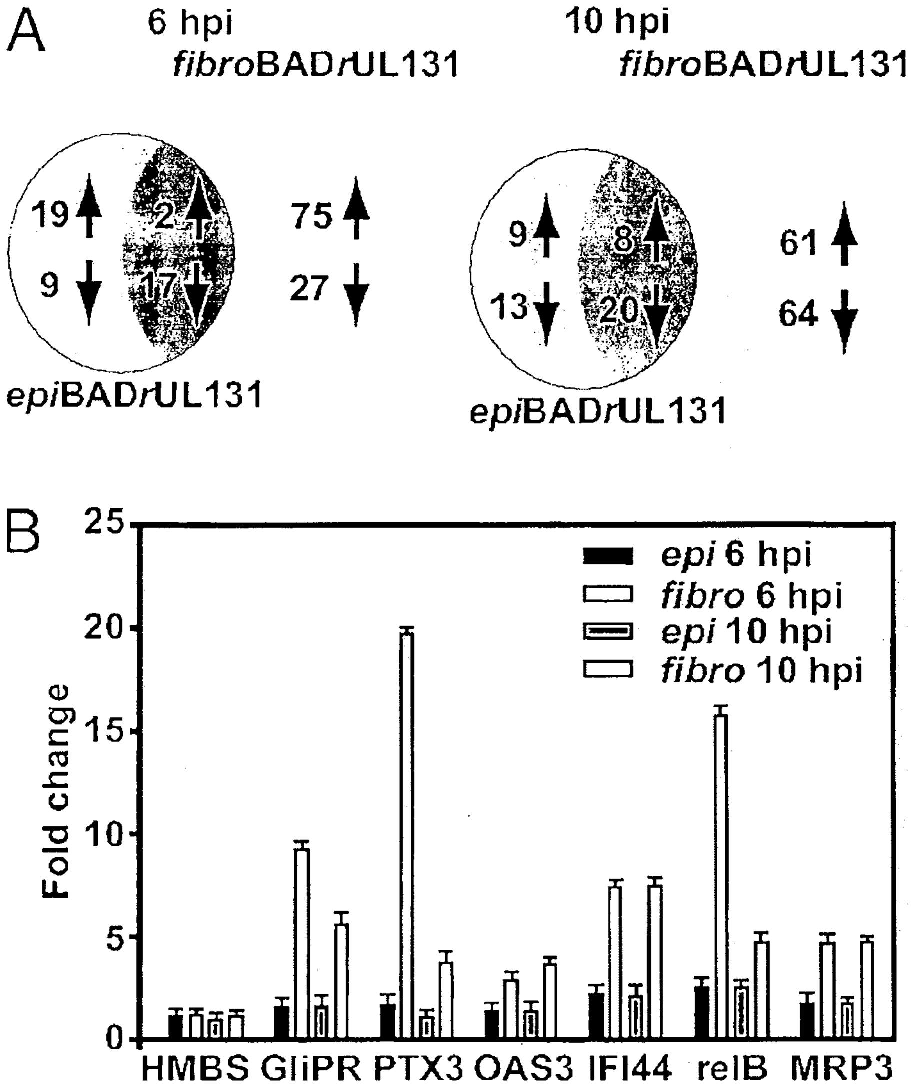
A
6 hpi
*fibro*BAD*r*UL131
10 hpi
*fibro*BAD*r*UL131
19
2
9
17
75
27
9
8
13
20
61
64
*epi*BAD*r*UL131
*epi*BAD*r*UL131
B
25
20
15
10
5
0
Fold change
epi 6 hpi
fibro 6 hpi
epi 10 hpi
fibro 10 hpi
HMBS
GliPR
PTX3
OAS3
IFI44
relB
MRP3

Fig. 6