In Vitro Characterization of Human Cytomegalovirus-Targeting Therapeutic Monoclonal Antibodies LJP538 and LJP539

Hetalkumar D. Patel,a Pavel Nikitin,a Thomas Gesner,a,b James J. Lin,a David T. Barkana, Claudio Ciferri,a,b Andrea Carfi,a* Tahmineh Akbarnejad Yazda, Peter Skewes-Cox,a Brigitte Wiedmann,a Nadine Jaroussa,b Weidong Zhong,a Adam Feire,a* Christy M. Hebnera

Novartis Institutes for BioMedical Research, Infectious Disease Area, Emeryville, California, USAa; Novartis Vaccines, Cambridge, Massachusetts, USAb

Human cytomegalovirus (HCMV) infection is usually benign in healthy individuals but can cause life-threatening disease in those with compromised immune systems. Approved drugs available to treat HCMV disease, including ganciclovir, cidofovir, and foscarnet, have significant toxicities that limit their use in certain patient populations. LJP538 and LJP539 are human monoclonal antibodies that are being evaluated as immunoglobulin therapeutics. The antibodies target glycoproteins gB and the gH/gl/UL128/UL130/UL131a pentameric complex, respectively. Here we present an in vitro characterization of these antibodies. We show that LJP538 and LJP539 are more potent than a marketed immunoglobulin at inhibiting HCMV infection of various cell lines relevant to pathogenesis. We find that LJP538 and LJP539 are active against a panel of clinical isolates in vitro and demonstrate minor-to-moderate synergy in combination. Passage of HCMV in the presence of LJP538 or LJP539 alone resulted in resistance-associated mutations that mapped to the target genes. However, no loss of susceptibility to the combination of antibodies was observed for >400 days in culture. Finally, the binding regions of LJP538 and LJP539 are conserved among clinical isolates. Taken together, these data support the use of LJP538 and LJP539 in combination for clinical trials in HCMV patients.

H uman cytomegalovirus (HCMV) can cause significant disease in immunocompromised individuals, including transplant recipients, those infected with HIV, and neonates infected in utero. Available HCMV therapies, such as ganciclovir (and its prodrug valganciclovir), cidofovir, and foscarnet, are effective but associated with serious toxicities. No therapy is available to prevent or treat congenital HCMV.

HCMV hyperimmune globulin (a polyclonal IgG preparation purified from human plasma pools) can be used to prevent infection and disease in some transplant recipients. It is safe and well tolerated, but it is less effective than other antiviral therapies. In solid-organ transplant recipients, the lower efficacy of hyperimmune globulin than ganciclovir or valganciclovir limits its use to selected high-risk situations (1). Treatment of hematopoietic stem cell transplant recipients with HCMV hyperimmune globulin is not recommended because of its limited efficacy and association with veno-occlusive disease (2). However, immunoglobulin or HCMV hyperimmune globulin is often added to ganciclovir or foscarnet when treating HCMV pneumonia. HCMV hyperimmune globulin has also been tested as a treatment for congenital HCMV. A prospective cohort study of pregnant women with primary HCMV infections showed that hyperimmune globulin was safe and that it appeared to reduce the incidence of congenital disease (3, 4). A follow-up randomized, placebo-controlled study of 123 pregnancies also showed a trend toward efficacy, although the benefit of this therapy was not statistically significant (5). More potent immunoglobulin preparations may better prevent and treat HCMV disease.

HCMV can infect a wide variety of cell types, including monocytes, endothelial or epithelial cells, smooth muscle cells, fibroblasts, stromal cells, neuronal cells, neutrophils, and hepatocytes (6). Entry into these various cell types is mediated by different HCMV glycoproteins. Glycoprotein B (gB) is required for entry into all physiologically relevant cell types, while gH and gL form two different complexes that mediate the infection of distinct cell populations. A pentameric complex consisting of gH, gL, UL128, UL130, and UL131a allows infection of myeloid, epithelial, and endothelial cells, whereas a three-member complex consisting of gH, gL, and gO is essential for entry into fibroblasts and likely mediates a preceding fusion process necessary for the infection of all cell types (7–9). This broad cellular tropism means that HCMV disease can occur in a variety of organs. Furthermore, infection of endothelial and hematopoietic cells appears to facilitate the systemic spread of virus, while infection of epithelial cells and fibroblasts seems to contribute to high-level replication (6).

LJP538 and LJP539 are human monoclonal antibodies that target gB and the gH/gl/UL128/UL130/UL131a pentameric complex, respectively. LJP538 and LJP539 are currently being developed as a combination (termed CSJ148) for the treatment of HCMV, and clinical evaluation for safety and efficacy in stem cell transplant patients is under way. Using these antibodies in combination has several advantages. First, the gB-targeting antibody LJP538 inhibits HCMV infection of all of the cell types tested,
whereas LJP539 may reduce systemic spread by targeting the pentameric complex. Second, although antibodies directed against gB correlate with neutralizing activity (10), the pentameric complex may be the major neutralizing determinant in natural infections and for hyperimmune globulin (11, 12). Targeting of both gB and the pentameric complex should therefore maximize control of HCMV in vivo. Finally, using the combination of antibodies may decrease the development of resistance.

Here we present an in vitro characterization of LJP538 and LJP539, confirming that they block viral infection and syncytium formation in culture. We demonstrate that LJP538 and LJP539 are more potent than a marketed immunoglobulin, Cytotect, at inhibiting HCMV infection of various cell lines and against a variety of clinical isolates in vitro. In addition, we show that passage of HCMV in the presence of LJP538 or LJP539 individually leads to mutations in the glycoprotein targets but that reduced susceptibility was not observed when the antibodies were used in combination.

MATERIALS AND METHODS

Antibodies and compounds. LJP538 and LJP539 (previously designated 7H3 and 4I22, respectively) were isolated from HCMV-infected human donors (13). These antibodies were synthesized at Novartis. Cytotect was acquired from Pavour Pharmaceuticals. A human anti-chicken lysozyme IgG1 control antibody was obtained from Abcam. Cetuximab is a chimeric mouse-human antibody with mouse Fab and human Fc portions that recognizes the human epidermal growth factor receptor (EGFR) (14). Cetuximab was purchased as Erbitux (lot 10C00002B; Eli Lilly) from McKesson. MSL-109 (synthesized at Novartis) is a human monoclonal IgG that recognizes a conformational epitope on gH (15). Ganciclovir was obtained from the laboratory of Thomas Shenk, Princeton University (see the supplemental material). The resulting virus, referred to here as AD169rUL131, was used for experiments.

Preparation of HCMV stocks. HCMV reference strain VR1814 was obtained from the American Type Culture Collection (ATCC). The AD169/UL131 reference strain was created at Novartis from parent strain AD169 (ATCC). As parent strain AD169 contains a mutation in UL131 that renders the virus incapable of forming a pentameric complex, a version of AD169 was created in which the UL131 mutation was reversed by using a pAD/Cre bacterial artificial chromosome (BAC) system that was a generous gift from the laboratory of Thomas Shenk, Princeton University (see the supplemental material). The resulting virus, referred to here as AD169/UL131, was used for experiments.

Reference strain viral stocks were produced by infection of adult retinal pigment epithelial (ARPE-19) cells or normal human dermal fibroblasts (NHDF) as previously described (16). Briefly, culture medium was removed and the cell monolayer (80 to 90% confluency) was rinsed with 10 ml of serum-free Dulbecco’s modified Eagle’s medium (DMEM)/F-12. Virus (0.3 to 1 ml) was mixed with 7 ml of serum-free medium and added to the cell monolayer at a multiplicity of infection (MOI) of 0.01. Cells were incubated at 37°C in 5% CO₂ for 20 to 50 days, and the medium was exchanged once per week until the appearance of plaques. Virus was harvested once the cytopathic effect (CPE) was complete. Cells were removed by scraping, and the mixture was sonicated for 1 hr at room temperature. Cells (80 to 90% confluent) were then inoculated with the virus-antibody complex and incubated for 3 h at 37°C in 5% CO₂. After inoculation, 7 ml of growth medium containing each antibody at a concentration equal to the EC₅₀ was added and cells were monitored for the appearance of a CPE. Virus was harvested from tissue culture medium by sorbitol pelleting once cell death was complete. In cases where virus replication was greatly reduced, as assessed by the inability of the virus to cause a complete CPE, the virus was harvested from the cells and supernatants after a maximum of 50 to 60 days in culture. Cells were lysed by sonication in an ice bath, and virus-containing supernatants were obtained after sedimentation of cellular debris. For passage in the presence of a combination of LJP538 and LJP539 in ARPE-19 cells, antibody concentrations corresponded to ~1 × EC₅₀ (0.648 µg/ml) of LJP538 and ~65 × EC₅₀ (0.0648 µg/ml) of LJP539. This dose of the antibodies was chosen to simulate the dose predicted to be efficacious in clinical trials. The cultures were maintained over 11 passages for 439 days.

The titer of the virus was determined and its antibody susceptibility was tested on the cell type that was used for resistance selection. If the virus was found to retain sensitivity to the selecting antibody, the infection protocol was repeated with gradually increasing amounts of antibody. Emergence of resistant virus was assessed by observation of a complete CPE within 2 to 3 weeks in the presence of antibody, at which point the virus was harvested.

RESULTS

LJP538 and LJP539 neutralize HCMV infection of various cell types. We tested the abilities of LJP538 and LJP539 to neutralize infection in cell types relevant to viral pathogenesis. Wild-type HCMV reference strain VR1814 was incubated with 10-fold dilutions of LJP538, LJP539, or Cytotect for 1 h and then used for infection. After 16 to 20 h of infection, cells were fixed and stained to detect IE1/2 proteins as a marker of HCMV infection. Both LJP538 and LJP539 neutralized HCMV strain VR1814 on the full range of physiologically relevant cell types (Table 1). In the two fibroblast types tested, the LJP538 EC₅₀ was 331- and 450-fold more potent than that of Cytotect. On epithelial and endothelial
TABLE 1 Neutralization of HCMV reference strain VR1814 infection in various cell types

| Cell type                      | LJP539 EC50 | EC90 | LJP539 EC50 | EC90 | Cytotect EC50 | EC90 |
|--------------------------------|-------------|------|-------------|------|---------------|------|
| Adult retinal pigment epithelial | 0.001       | 0.0052 | 0.84        | 2.37 | 4.56          | 13.81|
| Renal medullary epithelial      | 0.022       | 0.149 | 2.29        | 8.68 | 30.22         | 75.19|
| Renal cortical epithelial       | 0.004       | 0.012 | 0.68        | 2.33 | 12.54         | 31.84|
| Renal proximal tubule epithelial| 0.006       | 0.022 | 1.62        | 4.9  | 11.05         | 57.69|
| Placental epithelial            | 0.008       | 0.043 | 2.79        | 6.64 | 19.64         | 70.76|
| Uterine microvascular endothelial| 0.003      | 0.019 | 1.76        | 2.96 | 16.64         | 34.69|
| Human umbilical vein endothelial| 0.001       | 0.0083| 0.92        | 3.77 | 10.18         | 25.09|
| Human coronary artery endothelial| 0.005      | 0.009 | 0.86        | 3.01 | 2.54          | 18.92|
| Placental fibroblast            | >100        | >100  | 0.28        | 10.35| 22.13         | 3,432.99|
| Neonatal normal human dermal fibroblast | >100    | >100  | 1.4         | 2.81 | 280.3         | 1,264.35|

* Values are in micrograms per milliliter.

Characterization of LJP538 and LJP539

TABLE 2 Neutralization activity of LJP538 and LJP539 in combination against HCMV reference strain VR1814

| Cell type | Synergy vol | Antagonism vol | Synergy level |
|-----------|-------------|----------------|---------------|
| ARPE-19   | 46          | 0              | Minor         |
| HUVEC     | 74          | 0              | Moderate      |

* Synergy volumes at the 95% confidence level were calculated from eight replicates by using the MacSynergy II program and interpreted in accordance with the MacSynergy II manual (33).
days of culture with LJP539 was similar to that observed at 14 days of culture without the antibody. In the absence of the antibody or in the presence of a control IgG1 antibody, high titers of HCMV were seen at 7 to 14 days postinfection; titers decreased as the CPE overtook the culture (Fig. 2).

**LJP538 and LJP539 inhibit syncytium formation in HCMV glycoprotein-expressing cells.** In addition to viral entry from the supernatant, HCMV has the ability to spread directly from cell to cell through syncytium formation. Syncytia are conglomerates caused by fusion of cells expressing viral glycoproteins on their surface. To determine whether LJP538 and LJP539 could inhibit syncytium formation, we expressed HCMV glycoproteins in ARPE-19 cells and monitored fusion by high-content imaging. Six recombinant adenoviruses, each expressing HCMV glycoprotein gB, gH, gL, UL128, UL130, or UL131a, were used to transduce cells alone or in combinations (see the supplemental material). Western blotting showed that each adenovirus expressed the encoded HCMV envelope protein in an MOI-dependent manner (data not shown). The glycoproteins adopted physiologic conformations on the ARPE-19 cell surface, as shown by flow cytometry analysis with antibodies known to bind to conformational epitopes on gB and gH/gL/UL128/UL130/UL131a and through the ability of glycoprotein-expressing cells to block superinfection by VR1814 (data not shown). Syncytium formation was readily observed at 30 and 48 h posttransduction in cells expressing gB in combination with gH/gL/UL128/UL130/UL131a; other combinations of HCMV glycoproteins did not lead to cell-cell fusion (Fig. 3). Using the high-content assay, we showed that LJP538 and LJP539 inhibited cell-cell fusion at 30 h posttransduction with EC₅₀s of 4.77 and 0.076 μg/ml, respectively, while Cytotect was potent at 311.34 μg/ml (Table 3).

**LJP538, but not LJP539, shows a low yet detectable level of ADCC in vitro.** Antibody-dependent cell-mediated cytotoxicity (ADCC) is an immune defense mechanism whereby certain peripheral blood mononuclear cells (PBMC), in particular, natural killer (NK) cells, mediate cytotoxicity of antibody-coated microorganisms or host cells. Targeting of cells that express HCMV antigens for antibody-dependent destruction may be a benefit of immunoglobulin therapy by helping to deplete cells that are actively generating infectious virus. The possibility of LJP538- or LJP539-mediated ADCC in vitro was investigated by incubating ARPE-19 cells with antibodies and then coculturing them with PBMC from HCMV-seronegative donors (see the supplemental material). A low level of ADCC was observed in HCMV-infected cells in the presence of LJP538 (Fig. 4). A similar profile was observed with Cytotect. No ADCC was observed in the presence of LJP539 or in uninfected cells. A chimeric mouse-human antibody that recognizes the human EGFR, cetuximab, was used as a con-
trol and induced a high level of ADCC, as previously reported (14).

Passage of HCMV in the presence of monoclonal antibodies. HCMV strain VR1814 was passaged in ARPE-19 cells or NHDF in the presence of monoclonal antibodies. HCMV strain VR1814 was passaged in ARPE-19 cells or NHDF in the presence of LJP538 or LJP539 to investigate the emergence of resistance. Reduced susceptibility to the LJP538 antibody over four to six passages was seen in both NHDF and epithelial ARPE-19 cells and to LJP539 in ARPE-19 cells (Table 4); LJP539 was not tested in fibroblasts, as the pentameric complex is not required for entry into these cells. The reductions in susceptibility correlated with the detection of mutations in the coding regions of gB for LJP538 and UL130 for LJP539 (Table 4). By population sequencing, gB E361K and D362N mutations were detected in LJP538-selected virus from fibroblasts, while a deletion of gB amino acid E381 was observed in virus selected in epithelial cells. Sequencing of the coding region of UL130 in LJP539-selected virus from epithelial cells detected a Q191K mutation.

Viruses with reduced susceptibility to LJP538 and LJP539 in combination could not be isolated. Compared to the individual antibodies, passing in ARPE-19 cells in the presence of LJP538 and LJP539 in combination inhibited viral infection to a greater extent. This was indicated by a significant delay in the appearance of a CPE and much lower viral titers at each round of propagation (1 × 10^3 to 1 × 10^4 infectious units [IU]/ml) than typical for wild-type VR1814 (1 × 10^6 to 1 × 10^7 IU/ml). After 439 days in culture, titers were too low for the virus to be analyzed in the neutralization assay. However, we were able to PCR amplify gB, gH, gL, UL128, UL130, and UL131a; no mutations were detected (Table 4).

Engineering of resistance-associated mutations. We investigated the roles of the resistance-associated mutations by engineering each variant (UL130 Q191K, gB E361K, gB D362N, gB E361K D362N, and gB E381 deletion) into HCMV reference strain AD169rUL131 by using BAC mutagenesis. The wild-type and mutant viruses reached comparable titers in culture (data not shown). The abilities of LJP538 and LJP539 to neutralize the wild-type and mutant viruses were then compared (Table 5). When introduced individually into the HCMV genome, two of the gB mutations observed on selection with LJP538 were found to result in decreased susceptibility to the antibody (E361K and E381 deletion). In contrast, the single D362N mutation conferred no significant resistance. Both mutations together, however, reduced susceptibility more than the E361K mutation alone did. The UL130 Q191K mutation also resulted in a decrease in susceptibility to LJP539.

LJP538- and LJP539-resistant variant sites are conserved in clinical isolates. To investigate the natural occurrence of the mutations identified in vitro, 22 clinical isolates susceptible to neutralization by LJP538 or LJP539 (see Tables S1 to S3 in the supplemental material) were analyzed for the presence of the gB and UL130 amino acid mutations detected in passaged virus by Sanger sequencing and the dominant sequences were compared. The gB E361K, D362N, and E381 deletion mutations and the UL130 Q191K mutation were not detected by population sequencing in any of the HCMV clinical isolates tested. One conservative change (D to E at gB residue 362) was found in isolates 8816 and 8824. The UL130 region of one clinical isolate, MP-MD-805, could not be amplified.

The natural prevalence of these amino acid changes was also analyzed in sequences deposited in the NCBI database. Fifty-eight unique sequences of gB, 74 of UL130, and 12 complete HCMV genomes were aligned with corresponding gB and UL130 sequences. Two of these sequences had the conservative mutation D362E, while two had E361K. The UL130 Q191K mutation was not detected by population sequencing in any of the HCMV clinical isolates tested. One conservative change (D to E at gB residue 362) was found in isolates 8816 and 8824. The UL130 region of one clinical isolate, MP-MD-805, could not be amplified.

HCMV passaged in the presence of LJP538 or LJP539 remains susceptible to the nonselecting antibody. Pooled HCMV resistant to LJP538 after passage in the presence of antibody on...
NHDF was tested for neutralization by LJP538 on NHDF and LJP539 on ARPE-19 cells. As expected, the virus had reduced susceptibility to LJP538; however, it remained susceptible to LJP539 (Table 6). Similarly, pooled virus resistant to LJP538 after passage in epithelial cells was not as readily neutralized by LJP538 on NHDF and remained sensitive to LJP539 on ARPE-19 cells. Virus resistant to LJP539 after passage in epithelial cells showed decreased susceptibility to LJP539 on ARPE-19 cells but remained susceptible to LJP538 on ARPE-19 cells. These results indicate an absence of cross-resistance between LJP538 and LJP539, consistent with the antibodies targeting distinct glycoproteins.

Absence of cross-resistance between LJP538 or LJP539 and ganciclovir. We assessed the potency of ganciclovir against virus pools with decreased susceptibility to LJP538 or LJP539 generated in both NHDF and ARPE-19 cells. In all cases, both mutant and wild-type viruses remained susceptible to ganciclovir (see Table S5 in the supplemental material).

The resistance mechanism of LJP538 is different from that of MSL-109. MSL-109 is an IgG1 monoclonal antibody that targets HCMV glycoprotein gH and neutralizes infection in vitro (20). MSL-109 failed to prevent HCMV infection after allogeneic hematopoietic stem cell transplantation. Subsequent in vitro studies demonstrated that MSL-109 is subject to an unusual nongenetic escape mechanism in which the antibody is taken up by HCMV-infected cells and incorporated into the virion. The virus subsequently uses the Fc domain of the incorporated MSL-109 antibody to infect naive cells (16). This escape mechanism manifests itself in the acquisition of resistance within a single passage in cell culture and equally rapid reversion to the wild type after antibody withdrawal.

As candidates with a resistance mechanism similar to that of MSL-109 would not be clinically viable, LJP538 was experimentally compared to this antibody. We first investigated the ability of HCMV to rapidly escape neutralization on fibroblasts. Wild-type VR1814 virus was grown in the presence of MSL-109 (10 μg/ml) or LJP538 (10 μg/ml) in NHDF for a single passage. As previously observed (16), growth in the presence of the drug. Reduced susceptibility to ganciclovir in NHDF was confirmed, with an EC₅₀ shift of 23.74-fold (data not shown). Virus with decreased sensitivity to ganciclovir was then tested for the ability to be neutralized by antibodies (see Table S5 in the supplemental material). Both LJP538 and HCMV hyperimmunoglobulin (Cytotect) neutralized HCMV with decreased susceptibility to ganciclovir as potently as the wild-type virus. Parental AD169 virus does not express the pentameric complex and thus cannot infect ARPE-19 cells or be tested for susceptibility to LJP539.

### TABLE 4
| Antibody or combination | Cell type | No. of days in culture | No. of passages | Fold shift in EC₅₀ vs wild type | Protein; resulting mutation(s) |
|-------------------------|-----------|------------------------|----------------|-------------------------------|-------------------------------|
| LJP538-LJP539           | ARPE-19   | 439                    | 11             | _b                           | NDc                           |
| LJP538                  | NHDF      | 115                    | 6              | >41                           | gB; E361K, D362N               |
| LJP538                  | ARPE-19   | 158                    | 5              | 22                            | gB; E381 deletion             |
| LJP539                  | ARPE-19   | 76                     | 4              | >50,000                       | UL130; Q191K                  |

*Fold shifts were calculated by dividing antibody EC₅₀ for selected virus by those for wild-type VR1814. A greater-than sign before a fold shift value indicates that the antibody EC₅₀ for the selected virus was greater than the highest concentration tested.
_ = viral titer insufficient for assessment.
ND, none detected.
TABLE 5 Phenotypic characterization of mutations identified during resistance selection

| Antibody used during selection and protein (mutation[s]) | Cell type used for: | Fold shift in EC\textsubscript{50} relative to wild-type BAC-derived HCMV\textsuperscript{a} |
|--------------------------------------------------------|-------------------|--------------------------------------------------|
| LJP538 | ARPE-19 | NHDF | HUVEC |
| gB (E361K) | 19.1 | 1.0 | 9.5 | 140.9, 25.1 |
| gB (E381 deletion) | >195.5, >109.2 | >128.2, >149.7 | >284.7, >114.9 |
| gB (D362N) | 0.3 | 0.8 | 3.2 | 1.8, 2.0 |
| gB (E361K D362N) | 34.3 | >109.2 | >128.2, >149.7 | >284.7, >114.9 |
| LJP539, UL130 (Q191K) | >493.7, >405.7 | NA\textsuperscript{b} | >4,714.6, >1,475.1 |

\textsuperscript{a} EC\textsubscript{50} shift was calculated on the basis of the potency of the antibody indicated. Duplicate fold shift values are shown for each mutation. They were obtained from two independent neutralization assays done in triplicate.

\textsuperscript{b} NA, not applicable.

TABLE 6 Absence of cross-resistance between LJP538 and LJP539\textsuperscript{a}

| Antibody used for selection | Cell type used for: | Fold shift in potency relative to wild-type VR1814 for: |
|-----------------------------|-------------------|------------------------------------------|
|                             | Viral selection   | EC\textsubscript{50} generation | LJP538 | LJP539 |
| LJP538 | NHDF | ARPE-19 | ND\textsuperscript{b} | ND | 0.9 | 1.6 |
| LJP538 | NHDF | NHDF | >35.7 | >17.7 | NA\textsuperscript{c} | NA |
| LJP538 | ARPE-19 | ARPE-19 | 11.3 | 5.8 | 1.1 | 0.7 |
| LJP539 | ARPE-19 | ARPE-19 | 1.2 | 1.2 | >666.6 | >192.3 |

\textsuperscript{a} The neutralization potencies of LJP538 and LJP539 against wild-type VR1814 in ARPE-19 cells and NHDF are shown in Table 1.

\textsuperscript{b} ND, not determined.

\textsuperscript{c} NA, not applicable.
able to test all of the cell types that may be important to disease, these results suggest that the antibodies may be effective across multiple tissue types in vivo. LJP538 and LJP539 were also capable of neutralizing >20 clinical isolates. In all of the strains and cell types tested, LJP538 and LJP539 were more potent at neutralizing HCMV than hyperimmune globulin was. Clinical trials using HCMV hyperimmune globulin in pregnant women have shown a trend toward efficacy, suggesting that a more potent immunoglobulin may be beneficial in this population.

LJP538 and LJP539 were also tested in a long-term (28-day) infection experiment. LJP538 was capable of inhibiting infection at both 1× and 10× EC90 over the course of the experiment. While full inhibition was observed with LJP539 at 10× EC90, breakthrough occurred with this antibody at 1× EC90. Previously published data demonstrate that HCMV infection in endothelial and epithelial cell cultures is primarily focal, strictly cell associated, and highly dependent on the pentameric complex (23, 24). It is therefore possible that LJP539 is less effective at inhibiting the transmission of endothelial cell-tropic virus because of the stoichiometry of the pentameric complex, yet-to-be-understood biological differences in this transmission process, or limitation of antibody access. Since LJP538 is capable of inhibiting infection at the lower concentration, however, antibody access is unlikely to be an issue, consistent with a previous
phenomenon. As measured by syncytium formation, was examined by using ad- 
mixture at EC50s with 2- to 4-log greater potency than hyperim- 
were higher in the syncytium formation assay than in the neutral-

observation. Importantly, when HCMV was propagated 

mechanism of resistance similar to that of the clinically unsuccessful 

LJP539. Notably, in fibroblasts, LJP538 did not display a mecha-

(Q191K) resulted in a virus with decreased susceptibility to 

When introduced into the HCMV genome, a UL130 mutation 

reduced susceptibility to LJP538 than the E361K mutation alone. 

nation of the E361K and D362N mutations resulted in further 

When introduced into the HCMV genome, two of the gB mutations observed on selection with 

antibody remained effective against ganciclovir-resistant strains, in-

antibodies therefore have the potential for in vivo activity against a 

broad spectrum of naturally occurring HCMV strains. 

Cross-resistance studies indicated that LJP539 can neutralize 

HCMV with reduced susceptibility to LJP538, while LJP538 can 

neutralize HCMV with reduced susceptibility to LJP539 at con- 
centrations similar to those required to inhibit the wild-type virus. 

Although these data were not unexpected, considering that the antibodies target distinct glycoprotein complexes, the lack of 
cross-resistance is of clinical importance. Furthermore, each an-
tibody remained effective against ganciclovir-resistant strains, 
indicating that the antibody combination may be a useful therapy 
for ganciclovir-resistant HCMV infections. 

These antibodies will be administered in combination to pa- 

patients, which is important for multiple reasons. Disease pathogen- 

esis appears to require HCMV to infect different cell types. Infec-
tion of endothelial and hematopoietic cells may facilitate the 
systemic spread of the virus, while infection of fibroblasts may 
contribute to high-level replication (6). While earlier studies sug-
gested that epitopes within gB made up half of the neutralizing 
activity of human sera (10), more recent studies show that natu-

ically infected individuals make antibodies that inhibit epithelial 

epitopes at a significantly higher titer than those inhibiting fibro-

blast epitopes (29). Analysis of HCMV hyperimmune globulin 
showed that most of the neutralizing activity contained therein 
was directed against the pentameric complex, further highlighting 
the importance of antipentamer antibodies in controlling HCMV 
fection (12). While these data suggest that antibodies directed 
against the pentameric complex may be essential to viral control, 
the anti-gB antibody LJP538 has the added benefit of inducing some level of ADCC in vitro. Furthermore, animal models of con-
genital transmission of CMV (guinea pig, rhesus macaque), as 
well as human vaccine studies, demonstrated that inhibition of only one cell entry pathway is not sufficient to provide efficient 
protection (30, 31, 32). Neutralization of the two major cell entry 
pathways may therefore be more effective in reducing the HCMV 
load. In addition, in vitro studies suggest that the combination of 
antibodies may have a significantly higher barrier to resistance 
than each antibody alone. Taken together, these data support the 
continued development of LJP538 and LJP539 in combination for 
the prevention and treatment of HCMV infection. 

ACKNOWLEDGMENTS 

We thank Peter Pertel for critical review of the manuscript. We thank 
Catherine Jones for writing assistance. We thank Thomas Shenk, Mark
REFERENCES

1. Torres-Madrig F, Boucher HW. 2008. Immunocompromised hosts: perspectives in the treatment and prophylaxis of cytomegalovirus disease in solid-organ transplant recipients. Clin Infect Dis 47:702–711. http://dx.doi.org/10.1086/590934.

2. Hahn G, Revello MG, Patrone M, Percivalle E, Campanini G, Sarasini G, Adler SP, La Torre R, Best AM, Congenital Cytomegalovirus Study Group. 2012. Identification and expression of human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. J Virol 76:123–130. http://dx.doi.org/10.1128/JVI.01278-11.

3. Wang D, Shenk T. 2005. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. Proc Natl Acad Sci U S A 102:18153–18158. http://dx.doi.org/10.1073/pnas.0509201102.

4. Nokta M, Tolpin MD, Nadler PI, Pollard RB. 2014. How to trigger a killer: modulation of natural killer cell reactivity on many levels. Adv Immunol 124:137–170. http://dx.doi.org/10.1016/B978-0-12-800147-9.00005-4.

5. Ibig-Rehm Y, Gotte M, Gabriel D, Woodhall D, Shea A, Brown NE, Compton T, Feire AL. 2011. High-content screening to distinguish between attachment and post-attachment steps of human cytomegalovirus entry into fibroblasts and epithelial cells. Antiviral Res 89:246–256. http://dx.doi.org/10.1016/j.antiviral.2011.07.010.

6. Scharschmidt BF, Chau T, Cullen BR, Vries EL, Koff WC, Singher NA, Remick SG. 2014. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. Clin Cancer Res 1:1311–1318.

7. Nokta M, Tolpin MD, Nadler PI, Pollard RB. 1994. Human monoclonal anti-cytomegalovirus (CMV) antibody (MSL 109): enhancement of in vitro foscarnet- and ganciclovir-induced inhibition of CMV replication. Antiviral Res 24:17–26. http://dx.doi.org/10.1016/01663542(94)90048-5.

8. Boechtold B, Bowden R, Storer B, Chao NJ, Spielberger R, Tierney DK, Chandramouli V, Polilli E, Revello MG, Lazzarotto T, Guerra B, Spinillo A, Ferrazzi E, Kuster T, Feire AL. 2013. Neutralizing antibodies are unable to inhibit direct viral cell-to-cell fusion when expressed either in cis or in trans. J Virol 87:11837–11850. http://dx.doi.org/10.1128/JVI.01623-08.

9. Auerbach X, Meza BP, Adler SP, McVoy MA. 2008. Cytomegalovirus vaccines fail to induce epithelial entry neutralizing antibodies comparable to natural human antibody. Virology 444:140–147. http://dx.doi.org/10.1016/j.virol.2006.03.002.

10. Watzl C. 2014. How to trigger a killer: modulation of natural killer cell reactivity on many levels. Adv Immunol 124:137–170. http://dx.doi.org/10.1016/B978-0-12-800147-9.00005-4.

11. Atalay R, Zimmermann A, Wagner M, Borst E, Benz C, Messerle M, Hengel H. 2002. Identification and expression of human cytomegalovirus transcription units coding for two distinct Fcgamma receptor isoforms. J Virol 76:8596–8608. http://dx.doi.org/10.1128/JVI.76.17.8596-8608.2002.

12. Scharschmidt BF, Chau T, Cullen BR, Vries EL, Koff WC, Singher NA, Remick SG. 2014. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. Clin Cancer Res 1:1311–1318.

13. Nokta M, Tolpin MD, Nadler PI, Pollard RB. 1994. Human monoclonal anti-cytomegalovirus (CMV) antibody (MSL 109): enhancement of in vitro foscarnet- and ganciclovir-induced inhibition of CMV replication. Antiviral Res 24:17–26. http://dx.doi.org/10.1016/0166-3542(94)90048-5.

14. Manley K, Anderson J, Yang F, Szustakowski J, Oakley EJ, Compton T, Feire AL. 2011. Human cytomegalovirus escapes a naturally occurring neutralizing antibody by incorporating it into assembling virions. Cell Host Microbe 10:197–209. http://dx.doi.org/10.1016/j.chom.2011.07.010.

15. Ibig-Rehm Y, Gotte M, Gabriel D, Woodhall D, Shea A, Brown NE, Compton T, Feire AL. 2011. High-content screening to distinguish between attachment and post-attachment steps of human cytomegalovirus entry into fibroblasts and epithelial cells. Antiviral Res 89:246–256. http://dx.doi.org/10.1016/j.antiviral.2011.07.007.

16. Boechtold B, Bowden R, Storer B, Chao NJ, Spielberger R, Tierney DK, Chandramouli V, Polilli E, Revello MG, Lazzarotto T, Guerra B, Spinillo A, Ferrazzi E, Kuster T, Feire AL. 2013. Neutralizing antibodies are unable to inhibit direct viral cell-to-cell fusion when expressed either in cis or in trans. J Virol 87:11837–11850. http://dx.doi.org/10.1128/JVI.01623-08.

17. Auden SR, Oakeley EJ, Collinson P, Komorowski B, Brown NE, Komorowski B. 2012. Identification and expression of human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. J Virol 76:123–130. http://dx.doi.org/10.1128/JVI.01278-11.

18. Wang D, Shenk T. 2005. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. Proc Natl Acad Sci U S A 102:18153–18158. http://dx.doi.org/10.1073/pnas.0509201102.

19. Watzl C. 2014. How to trigger a killer: modulation of natural killer cell reactivity on many levels. Adv Immunol 124:137–170. http://dx.doi.org/10.1016/B978-0-12-800147-9.00005-4.
31. Schleiss MR, Buus R, Choi KY, McGregor A. 2013. An attenuated CMV vaccine with a deletion in tegument protein GP83 (pp65 homolog) protects against placental infection and improves pregnancy outcome in a guinea pig challenge model. Future Virol 8:1151–1160. http://dx.doi.org/10.2217/fvl.13.107.

32. Pass RF, Zhang C, Evans A, Simpson T, Andrews W, Huang ML, Corey L, Hill J, Davis E, Flanigan C, Cloud G. 2009. Vaccine prevention of maternal cytomegalovirus infection. N Engl J Med 360:1191–1199. http://dx.doi.org/10.1056/NEJMoa0804749.

33. Prichard MN, Aseltine KR, Shipman CJ. 1993. MacSynergy II manual. University of Alabama at Birmingham, Birmingham, Alabama. http://www.uab.edu/images/pediatrics/ID/MacSynergy.pdf.