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A High-Affinity Native Human Antibody Neutralizes Human Cytomegalovirus Infection of Diverse Cell Types

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Human cytomegalovirus (HCMV) is the most common infection causing poor outcomes among transplant recipients. Maternal infection and transplacental transmission are major causes of permanent birth defects. Although no active vaccines to prevent HCMV infection have been approved, passive immunization with HCMV-specific immunoglobulin has shown promise in the treatment of both transplant and congenital indications. Antibodies targeting the viral glycoprotein B (gB) surface protein are known to neutralize HCMV infectivity, with high-affinity binding being a desirable trait, both to compete with low-affinity antibodies that promote the transmission of virus across the placenta and to displace nonneutralizing antibodies binding nearby epitopes. Using a miniaturized screening technology to characterize secreted IgG from single human B lymphocytes, 30 antibodies directed against gB were previously cloned. The most potent clone, TRL345, is described here. Its measured affinity was 1 pM for the highly conserved site I of the AD-2 epitope of gB. Strain-independent neutralization was confirmed for 15 primary HCMV clinical isolates. TRL345 prevented HCMV infection of placental fibroblasts, smooth muscle cells, endothelial cells, and epithelial cells, and it inhibited postinfection HCMV spread in epithelial cells. The potential utility for preventing congenital transmission is supported by the blockage of HCMV infection of placental cell types central to virus transmission to the fetus, including differentiating cytotrophoblasts, trophoblast progenitor cells, and placental fibroblasts. Further, TRL345 was effective at controlling an *ex vivo* infection of human placental anchoring villi. TRL345 has been utilized on a commercial scale and is a candidate for clinical evaluation.

Human cytomegalovirus (HCMV) is the most common medically significant infection in transplant patients (1). It is also the leading cause of congenital viral infection, with an incidence in the United States of 1 to 3% of live births. Primary HCMV infection during early pregnancy poses a 40 to 50% risk of intrauterine transmission. Approximately 5 to 10% of congenitally infected infants are symptomatic, presenting with intrauterine growth restriction and permanent birth defects, including neurological deficiencies, retinopathy, and sensorineuronal deafness (2–4). Becoming infected at an early gestational age increases the severity of these problems (5). Even secondary reactivations or reinfections of seropositive (previously exposed) women can lead to birth defects if the virus is passed on to the fetus (6). Intrauterine growth restriction imposes significant medical costs at delivery and is associated with cardiovascular disease later in life (7). Further, up to 15% of stillbirths (death *in utero* after 20 weeks gestation) are associated with congenital HCMV (8, 9), and infection of the cervix can reduce resistance to bacterial infections, leading to preterm labor (10). Conversely, the administration of multiple doses of hyperimmune globulin (HIG) after primary maternal infection in early gestation may enhance birth weight and gestational age at delivery (11).

To date, vaccination to prevent HCMV infection has not been proven to provide adequate protection against maternal infection, reinfection, or fetal transmission (12, 13), and it would be difficult to implement in immunosuppressed transplant patients. *Ex vivo* stimulation of T cells with HCMV antigens has shown promise as an alternative to traditional vaccination (14), but this technology is cumbersome to implement on a commercial scale. Moreover, the antiviral activity of T cells is inhibited by granulocyte colony-stimulating factor (G-CSF) (15), which is commonly used to promote the engraftment of hematopoietic stem cells.

An alternative to traditional vaccines is to administer HIG, which for decades has been used safely in pregnancy to treat blood group incompatibilities, rubella, hepatitis, varicella, and measles (16). Passive immunization with HIG produced from HCMV-seropositive donors has shown promising results in human clinical trials (17–21), although the degree of efficacy remains controversial. A recent study (22) observed that HIG failed to meet the trial's primary endpoint of preventing fetal infection; however, the study was underpowered, and a meta-analysis of the data combined with another study revealed that efficacy was highly likely ($P < 0.05$) (23).

Monoclonal antibodies (MAbs) of diverse types have replaced HIG for other diseases (24, 25), reflecting several advantages:

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MAbs provide more consistency in manufacturing, with a reduced risk of contamination by human viruses or prions, and they have a reduced potential for adverse events arising from off-target reactivity and higher specific activity that enables lower doses and simpler administration.

HCMV has a double-stranded DNA genome of 235 kb, with approximately 200 potential protein-coding regions, including 20 proteins associated with the virion envelope. These and other proteins produced by infected cells modulate a complex interaction with the immune system to protect the virus, providing multiple potential targets for antibody attack (26). Several reports have described human MAbs that neutralize HCMV (13). One major group of antibodies is directed toward the gB protein, a key component of subunit vaccines. Recombinant gB formulated with MF59 adjuvant was found to be 50% effective in preventing primary infections, supporting the identification of gB as a promising target (27). The failure to induce more uniform efficacy may relate to the fact that the invariant epitope on gB (AD-2, site I) is poorly immunogenic (28, 29). The MAb ITC-88 has been a well-known example of this group for 2 decades (30). Another extensive group of published MAbs is directed against a set of virion envelope proteins named the pentameric complex (gH, gL, UL128, UL130, and UL131) (31, 32). This complex appears to be particularly relevant to infection in specialized host cells, including epithelial and endothelial cells and monocytes (33). Anti-pentamer antibodies are abundant in the commercial HIG product Cytotect (34) and are made early in the course of natural infection (35). A drawback to targeting the pentameric complex is that most antibodies against it fail to neutralize infections in fibroblasts (36), an important cell type for disease progression. An MAb against gH has shown protection in a broader set of cell types, but escape mutants were readily obtained in <10 passages of the virus in the presence of the MAb (37).

Affinity is also an important consideration in developing a protective MAb against HCMV. Weak affinity is associated with enhanced fetal transmission (38), possibly because only high-affinity (low off rate) MAbs can prevent placental infection by remaining bound to the virus following transcytosis across syncytiotrophoblasts via the neonatal Fc receptor (39). The variabilities in affinity for different antigens may also result in variable efficacy of different batches of HIG. For MAbs targeting gB, affinity is particularly important, since competitive binding between neutralizing and nonneutralizing antibodies is a normal part of the natural immune response to gB, as was first established using murine hybridomas; the neutralizing MAb 7-17 and a nonneutralizing MAb, 27-287, were shown to bind competitively to gB, although at a different site from that of TRL345 (40). The high affinity of the neutralizing MAb provides protection from such interference by weaker-affinity MAbs (28). This aspect of HCMV structure is not a minor issue, as nonneutralizing antibodies to the nearby site II on gB(AD-2) were found in ~25% of anti-HCMV human serum samples (41). These features pose an additional obstacle to vaccine development and to quality control in HIG manufacturing.

We used our previously described single-B-cell screening platform (42, 43) to clone 30 different native human MAbs that bind to the highly conserved AD-2 (site I) epitope on gB (44). Although TRL345 was not the highest-affinity MAb, it had the best potency *in vitro* (44), substantially higher than that of either HIG or ITC-88, a well-studied MAb against this epitope (30). Its properties, both biological and biochemical, are described in detail here. Based on these results, TRL345 is being prepared for clinical development as a single-agent therapeutic to suppress or significantly reduce HCMV infection and related pathologies in a broad range of human cell types.

MATERIALS AND METHODS

Cells and viruses. Human MRC-5 fibroblasts (ATCC CCL-171) and ARPE-19 epithelial cells (ATCC CRL-2302) were obtained from the ATCC and propagated in high-glucose Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 10,000 IU/liter penicillin, and 10 mg/liter streptomycin (Gibco-BRL). Human umbilical vein cells (HUVECs) and smooth muscle cells were purchased from Lonza. As previously described, human placental fibroblasts (HPF) (45) and trophoblast progenitor cells (TBPCs) (46, 47) were isolated and cultured. Human foreskin fibroblasts (HFF) were isolated and propagated by conventional methods (48).

The virus BAD*r*UL131-Y4 (BADr) is a variant of HCMV strain AD169, in which a mutation in *UL131* has been repaired to express a functional UL131 protein (49). TS15-rN and TS15-rR are epithelial tropic variants of HCMV strain Towne (50). BADr, TS15-rN, and TS15-rR each encode an expression cassette for green fluorescent protein (GFP) and were amplified in ARPE-19 cultures. Frozen stocks were prepared from ARPE-19 culture supernatants and the titers determined, as described previously (51). VR1814 was propagated in HUVECs, followed by a single passage in HFF to obtain high-titer stock virus, as previously described (47). HCMV clinical isolates were cultured as described previously (52) from urine samples from 0- to 18-month children attending daycare in the Richmond or Norfolk, VA, areas. The isolates were passaged 3 to 4 times in MCR-5 cells before use in the neutralization studies; the isolates were gB typed by PCR-restriction digest analysis, as described previously (53).

Antibodies. Blood samples (Stanford Blood Center, Stanford, CA) were obtained from anonymized donors under informed consent approved by Stanford's institutional review board and screened by enzyme-linked immunosorbent assay (ELISA) for reactivity to an AD-2 peptide (NETIYNTTLKYGD). Memory B cells from the individuals with the highest titers were stimulated to divide using a mixture of mitogens and cytokines, and then these were screened at the single-cell level, as previously described (42). Briefly, this multiparameter assay enabled a concurrent measurement of binding to (i) the peptide with biotin and a hydrophilic PEG6 spacer attached to the N or C terminus (Genemed Synthesis, San Antonio, TX) at both high and low density on neutravidin-derivatized fluorescent latex beads and (ii) the human gB protein, which was prepared using an expression vector from Sino Biological (catalog no. V08H1; Beijing, China) encoding the extracellular domain of human gB (GenBank accession no. AAA45920.1; Met1-Lys700 linked with the cytoplasmic domain Arg777-Val907, with a furin cleavage site mutated from RTKR to TTQT, fused with a polyhistidine tag at the C terminus). Also, a lack of binding to bovine serum albumin (BSA) was used as a specificity counterscreen. After identifying a rare human B cell secreting an MAb meeting the selection criteria, the encoding mRNA was cloned by single-cell reverse transcription-PCR (RT-PCR) from the sibling cells of the originally identified positive cell. After subcloning into the pTT5 vector (under license from the National Research Council of Canada, Ottawa, Canada) (54), recombinant antibodies were transiently expressed in HEK293 FreeStyle cells from Invitrogen (Carlsbad, CA).

TRL345 has also been expressed in stably transformed CHO cells at 1.8 g/liter using the GPEx technology (Catalent Pharma Solutions, Madison, WI). The bulk product was clarified using Cuno depth filtration, followed by 0.2-μm filtration (Pall Corporation., Cortland, NY) before protein A purification (MabSelect column; GE Healthcare Life Sciences, Pittsburgh, PA), low pH to inactivate potential virus contaminants, and polishing by ion exchange (Mustang Q filtration; Pall Corporation) and hydroxyapatite chromatography (ceramic hydroxyapatite; Bio-Rad, Richmond, CA).

The resulting product was analyzed by size exclusion chromatography (SEC) using a Bio SEC-3 column (Agilent, Santa Clara, CA) of 4.6 mm by 30 mm, with a flow rate of 0.35 ml/min using 150 mM sodium phosphate buffer, and absorbance at 220 nm was monitored.

Synagis (palivizumab) was obtained from Medimmune, LLC (Gaithersburg, MD). Human anti-gB MAb ITC-88 (30) was a gift from Mats Ohlin (Lund University, Lund, Sweden). The anti-pentameric complex MAb TRL310 was constructed based on DNA sequences reported for antibody 1F11 (36), and it was expressed (54) and purified as described above. Human MAb TRL298 was isolated as above using beads coated with gB protein.

Epitope mapping. AD-2-related peptides were synthesized and high-performance liquid chromatography (HPLC) purified by GenScript (Piscataway, NJ). Eleven peptides were generated by stepwise truncation of the C terminus and 12 by stepwise truncation of the N terminus. The peptides were dissolved in dimethyl sulfoxide (DMSO), diluted to 2 μg/ml in Dulbecco's phosphate-buffered saline (PBS) (Mediatech, Manassas, VA), and passively coated onto ELISA microplates overnight at 4°C. After blocking with 3% BSA in PBS, TRL345 was applied to duplicate wells at 1 μg/ml in PBS for 1 h. Secondary horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA), followed by 3,3′,5,5′-tetramethylbenzidine (TMB) (HRP substrate), was used to measure bound TRL345 using standard methods (55).

***K_D* measurement.** TRL345 (stock at 600 nM) was diluted to 5.0 and 0.5 nM in PBS (pH 7.4) and passed across a ForteBio Octet biosensor model QK (Pall Corporation, Menlo Park, CA) with streptavidin-derivatized sensors coated with C-terminal biotinylated AD-2 peptide HRANE TIYNTTLKYGD-biotin (1 mg/ml in PBS [pH 7.4]) at a concentration of 250 nM, and the resulting on and off rates were measured to yield the K_D (equilibrium dissociation constant) using standard methods (56).

Neutralization and spread inhibition assays. The neutralization of HCMV entry into HUVECs was measured using the virus VR1814, and the cells were infected at a multiplicity of infection (MOI) of 0.1, as described previously (47). GFP-based neutralization and spread inhibition assays were conducted as described previously (33, 50, 57, 58). The neutralization of clinical isolates was determined by incubating viral stocks with TRL345 at a final concentration of 2.4 μg/ml (14.4 nM) for 1 h at 37°C and then adding the mixture to confluent MRC-5 cells in 24-well plates. The cultures were read visually for cytopathic effect at 15 days postinfection. For a comparison of TRL345 to HIG, MRC-5 cells were similarly infected with GFP-expressing BADr virus mixed with the antibodies at serial 2-fold dilutions starting from equipotent concentrations of TRL345 (12.5 μg/ml) and HIG (625 μg/ml), using GFP intensity as the readout for infection at 5 days postinfection.

Human placental villus explant model. Approval for this project was obtained from the institutional review board at the University of California, San Francisco. As previously described (59), chorionic villi dissected from human placentas at 6 to 8 weeks gestational age were cultured on Millicell-CM inserts (0.4-μm pore size; Millipore, Billerica, MA) coated with Matrigel (BD Biosciences, Bedford, MA) in explant medium (DMEM-F12 [1:1] [Gibco, Carlsbad, CA] with 10% HyClone fetal bovine serum [FBS] [Thermo Scientific, Logan, UT], 1% penicillin-streptomycin, and 1% mixed amino acids). After 18 to 20 h, diluted MAbs (final concentration, 1 or 10 μg/ml) or HIG (Cytotect; Biotest, Boca Raton, FL, final concentration 10 or 100 μg/ml) were incubated with HCMV VR1814 (3×10^6 PFU/explant) for 1 h at 37°C, with agitation. Note that for a full-length MAb, 1 μg/ml is equivalent to a 6 nM concentration. The virus-antibody mixtures were allowed to adsorb to cells for 2 h, washed with PBS, and supplemented with fresh growth medium. The explants were maintained for 3 days postinfection, harvested and fixed in 4% paraformaldehyde (Wako Chemical USA, Richmond, VA), equilibrated in 15% sucrose, embedded in 10% gelatin, frozen in dry ice, and cut into 5-μm sections for histological analysis. The blocks were sectioned and stained with the rat anti-cytokeratin monoclonal antibody 7D3 (60) (gift from Susan Fisher) to label the cytotrophoblasts and with anti-HCMV immunoelectrophoresis 1 (IE1) antigen (CH443) (produced in the Pereira lab) to identify infected cells. Primary antibodies were detected using fluorescently labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Infectivity was quantified by counting the number of cytokeratin-positive cells and the number of IE1-positive cells in individual cell columns by fluorescence microscopy using a Nikon Eclipse 50i microscope equipped with a Spot 7.4 slider digital camera and calculating the percentage of cytokeratin-positive cells that were infected. Each experimental condition was repeated three times. The significance of the differences between the experimental conditions was determined using a two-tailed Student's *t* test.

Stability assays. Aliquots of TRL345 (100 μl at 2 μg/ml) were heated for 10 min at selected temperatures ranging from 20 to 90°C using a StepOne Plus PCR instrument (Life Technologies, Inc., Carlsbad, CA), after which the samples were immediately pipetted into ELISA plates precoated with AD-2 peptide (HRANETIYNTTLKYGD; GeneMedSyn, Inc., San Antonio, TX); following incubation at room temperature for 1 h, bound antibody was quantified as in the epitope mapping experiments.

Pharmacokinetics. TRL345 was administered intravenously (i.v.) as a bolus injection at 10 mg/kg via the tail vein to three cannulated (jugular vein) male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) following acclimation at Murigenics, Inc. (Mare Island, CA). A baseline blood sample of 200 μl was collected via the cannula before dosing and then at 5 min, 1, 4, 8, 12, 24, 48, and 168 h after dosing. The serum samples were collected and stored at below −60°C until analysis. The study was performed after approval by the Murigenics Institutional Animal Care and Use Committee.

TRL345 in rat serum was quantified by ELISA following capture from the sample matrix via binding to immobilized AD-2 peptide, as in the stability assay described above. A goat anti-human kappa light-chain antibody conjugated to HRP (Sigma, St. Louis, MO) was used to detect the captured TRL345, using TMB substrate. The minimum quantifiable concentration in 100% rat plasma was 137 ng/ml.

An open two-compartment model was used to describe the elimination profile of TRL345 and to estimate the mean maximum concentration of drug in serum (C_{max}) (in mg/ml), the time to C_{max} (T_{max}) (in h), the distribution-phase half-life ($t_{1/2\alpha}$) (in h), the elimination-phase half-life ($t_{1/2\beta}$) (in h), the serum concentration at time zero (C_0) (in ng/ml), the apparent volume of distribution (V) (ml/kg), the apparent volume of distribution at steady state (V_{ss}) (in ml/kg), clearance (CL) (ml/h/kg), the area under the concentration-time curve from time zero to the last observed concentration (AUC_{0-t}) (in ng · h/ml), and the AUC from time 0 to infinity ($AUC_{0-\infty}$) (in ng · h/ml).

Tissue reactivity profile. Sections of fresh-frozen tissues from 3 tissue donors for each tissue type (Charles River Laboratories Pathology Associates, Reno, NV) or control cells (HEK293 cells transiently transfected with gB, or mock transfected) were cut on a cryostat, mounted onto slides, and fixed in cold acetone (at −10°C) for 10 min; these were then used immediately or stored at −80°C (and thawed overnight at room temperature before use). The tissues were qualified via staining with an antibody against CD31 (platelet endothelial cell adhesion molecule).

A staining complex was formed by incubating TRL345 or control antibody (human IgG1 kappa; Sigma, St. Louis, MO) with a biotinylated mouse anti-human IgG secondary antibody (Sigma) in a 1:1.5 ratio for 1 h, followed by blocking unbound secondary antibody with excess IgG for 30 min.

The slides were incubated in Morpho Save (Ventana Medical Systems, Tucson, AZ) for 15 min and then washed twice in PBS for 5 min. To block endogenous peroxidase, the slides were incubated in 20 mM glucose, 2 mM sodium azide, and 2 units/ml of glucose oxidase in PBS for 60 min at 37°C and then washed twice in PBS for 5 min. Endogenous biotin was blocked with streptavidin. The tissue sections were further blocked with blocking reagent (Covance, Dedham, MA) for 30 min. Staining com-

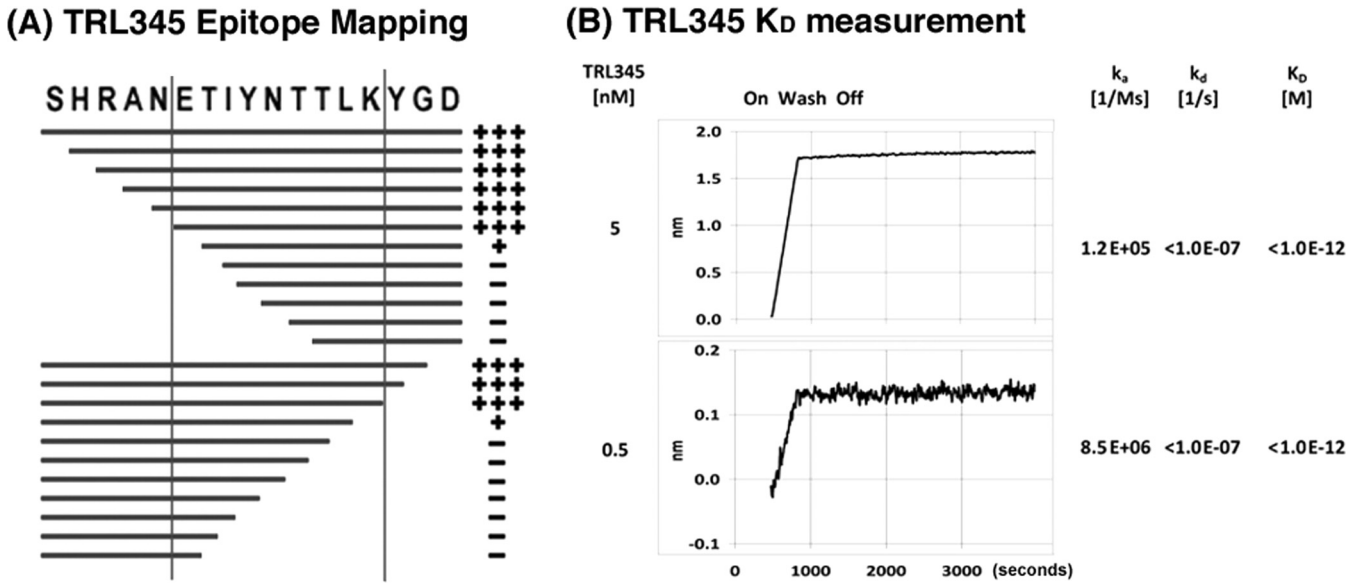

FIG 1 Binding specificity and affinity of TRL345. (A) Synthetic peptides spanning the AD-2 (site I) region of HCMV gB were immobilized on polystyrene microplates, and the binding of TRL345 was semiquantitatively scored by ELISA as strong (+++), moderate (+), or negative (−). (B) Kinetics of binding of TRL345 to the AD-2 peptide (HRANETIYNTTLKYGD-biotin) immobilized on a streptavidin-coated sensor was measured using a ForteBio Octet biosensor.

plexes were applied and incubated for 30 min. Bound antibody was visualized with a streptavidin-horseradish peroxidase (ABC) conjugate and a 3-3′-hydrochloric acid (HCl) diaminobenzidine (DAB) chromogen substrate (Sigma).

RESULTS

Screening technology. The CellSpot platform (42, 43) uses digital microscopy to examine the secreted IgG footprint (~100 fg per cell over a 4-h period) from each of millions of individual memory B cells. Following stimulation with a mixture of mitogens and cytokines, ~70% of resting memory B cells begin a course of proliferation and antibody secretion that lasts 10 days. Antigens, including both recombinant gB protein and a synthetic 13-mer peptide that includes the canonical AD-2 epitope sequence (61, 62), were conjugated to distinguishable fluorescent beads and used as probes of binding specificity. The assay was biased for high-affinity MAbs by varying the density of the peptide on distinguishable beads, thereby modulating the avidity boost associated with multidentate binding. A fourth bead type was coated with BSA as a specificity counterscreen. Of the 48 blood donors surveyed, we focused on 4 with a high titer by ELISA to the AD-2 peptide. Even in this group, the frequency of B cells secreting an MAb meeting all of the screening criteria was <1 in 100,000. From 2.5 million B cells surveyed from the donor with the highest frequency of anti-AD-2 B cells, 30 MAbs were cloned by single-cell cDNA PCR and expressed in HEK293 cells. As previously described, the V_H and V_L germ line usage in this set of MAbs was restricted, including both published and previously unidentified gene families against this target (44). Based on its superior potency for neutralizing HCMV *in vitro* (44) compared to that of the other MAbs cloned in the same manner, TRL345 was chosen for further characterization.

Biochemical assays. TRL345 binding was measured by ELISA against a set of overlapping sequence peptides derived from the AD-2 sequence, deleting residues from either the N or C terminus. Because all of the peptides may not adsorb equally to the ELISA plate, the pattern of binding was evaluated semiquantitatively. A strong signal was seen for the linear epitope (residues 69 to 77; ETIYNTTLK) corresponding to the highly conserved AD-2 (site I) region (61, 62) (Fig. 1A), with signal declining outside that motif. This epitope was searched by BLAST; of 80 HCMV sequences in the database, only 2 substitutions were found, T74 to S or A, which represent conservative substitutions. The affinity of TRL345 for the AD-2 peptide, with a K_D of 1 pM determined by biosensor analysis, arises from a low off rate (Fig. 1B).

Protection of multiple cell types. In the neutralization assays using HCMV strain VR1814 (62), TRL345 was effective in preventing HCMV infection in human endothelial cells, smooth muscle cells, placental fibroblasts, and trophoblast progenitor cells (Fig. 2A to D). In comparison, although the anti-pentameric complex antibody TRL310 had more potent neutralizing activity on endothelial cells than that of TRL345, consistent with expectations from published data on an MAb with the same sequence (36), TRL310 provided no protection against infection in smooth muscle cells, placental fibroblasts, or trophoblast progenitor cells (Fig. 2A to D). The neutralizing potency of an antiviral MAb is commonly reported as the antibody concentration that reduces viral entry by half (50% effective concentration [EC_{50}]). For protecting a wide range of cell types from infection by strain VR1814, the potency was approximately 0.1 μg/ml (0.6 nM) (Table 1). In side-by-side testing, TRL345 was 5-fold more potent than ITC-88, an extensively characterized MAb against gB, for which the published affinity is 2 nM (30) (Fig. 2E), and 50-fold more potent than the HIG product Cytotect (Fig. 2F). Similar potency was observed using MRC-5 human lung fibroblasts and ARPE-19 epithelial cells challenged with GFP-tagged epithelial-tropic variants of the laboratory strains Towne (TS15-rN) and AD169 (BADr), respectively (Table 1). Figure 2F also shows that other antibodies present in Cytotect did not interfere with the efficacy of TRL345 in MRC-5

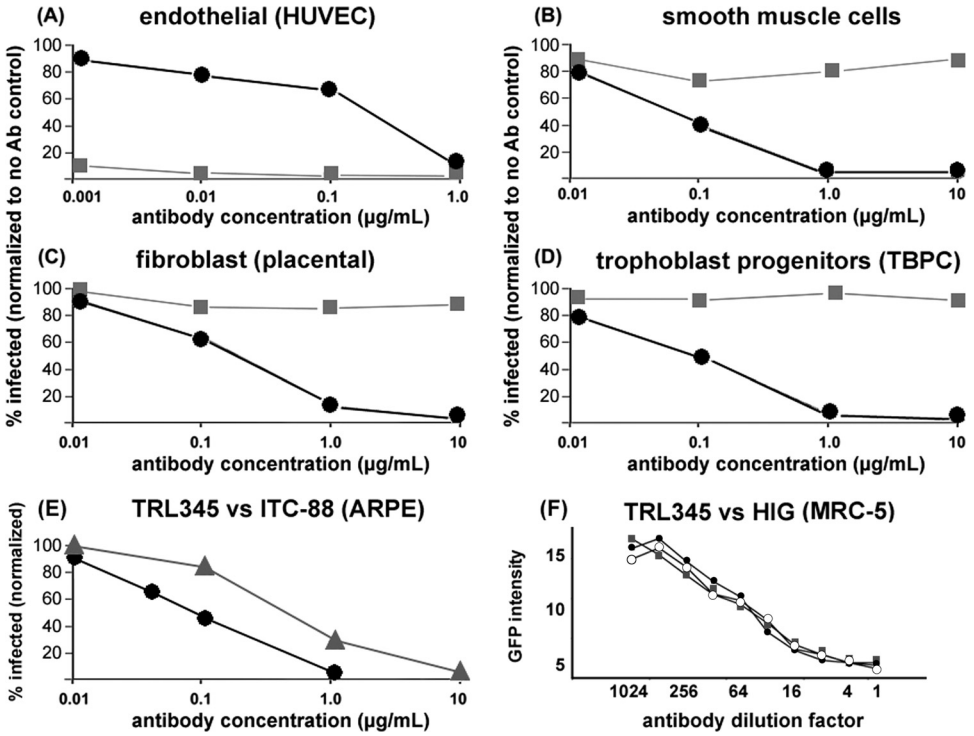

FIG 2 Neutralizing activity of TRL345. Serial dilutions of anti-gB MAb TRL345 or comparator antibodies were incubated with HCMV strain VR1814 and then added to cultures containing HUVECs (A), human smooth muscle cells (B), human placental fibroblasts (C), human trophoblast progenitor cells (D), adult retinal pigmented epithelial cells (E), and MRC-5 fetal lung fibroblasts (F). (A to E) Black circles, TRL345; gray squares, TRL310; gray triangles, ITC-88. The percentages of infected cells were determined by counting IE1-positive cells and normalizing to the counts from the no-antibody control. (F) Gray squares, HIG, starting from an initial concentration of 625 μg/ml; black circles, TRL345, starting from an initial concentration of 12.5 μg/ml; open circles, 1:1 mixture of TRL345 and HIG. The GFP intensity from infected cells was quantified at 5 days postinfection. Note that for a full-length MAb, 0.1 μg/ml is equivalent to a 0.6 nM concentration.

cells; when the two antibody preparations were mixed 1:1 from concentrations providing equal potency (625 μg/ml for HIG and 12.5 μg/ml for TRL345), the potency was unchanged across subsequent serial 2-fold dilutions.

Protection against infection with diverse clinical isolates. While the epitope recognized by TRL345 is highly conserved, it is possible that sequence polymorphisms elsewhere in the gB protein alter its conformation and thereby influence the ability of TRL345 to bind its epitope. To determine if TRL345 was effective against CMV strains with diverse gB genotypes, neutralization assays were conducted using 15 primary clinical isolates of diverse genotypes (62). TRL345 prevented infection in MRC-5 fibroblasts by all 15

TABLE 1 Potency of TRL345 for neutralizing viral entry into multiple cell types[a]

Cell type by virus strain	EC_{50} (μg/ml)
VR1814[b]	
HUVECs	0.42
ARPE-19	0.12
Smooth muscle cells	0.10
HFF	0.17
Placental cytotrophoblasts	0.20
Placental trophoblast progenitor cells	0.17
Placental fibroblasts	0.12
TS15-rN (Towne)[c]	
MRC-5 (human lung fibroblasts)	0.23
ARPE-19	0.08
BADr (AD169)[c]	
ARPE-19	0.07

[a] Note that for a full-length MAb, 0.1 μg/ml = 0.6 nM.
[b] Immunoelectrophoresis (IE) staining-based neutralizing assays.
[c] GFP-based neutralizing assays.

TABLE 2 Protection by treatment *in vitro* with TRL345 of MRC-5 fibroblasts from infection by clinical isolates of diverse genotypes

Isolate	gB serotype group	FFU with no antibody[a]	FFU with TRL345
CSL001	1	6	0
32524	1	4	0
32692	1	4	0
32583	2	4	0
32691	2	2	0
32195	2	2	0
32613	3	5	0
32635	3	4	0
CLS006	3	3	0
32516	3	3	0
32493	3	2	0
32514	3	2	0
UXCM	ND[b]	5	0
32648	ND	2	0
32552	ND	1	0

[a] Focus-forming units (FFU) were counted at 10 to 12 days postinfection.
[b] ND, not determined.

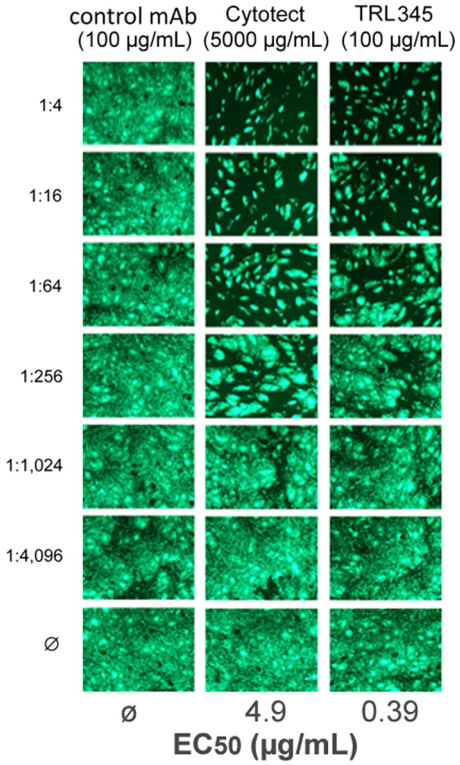

FIG 3 HCMV spread in ARPE-19 epithelial cells treated with TRL345. ARPE-19 cultures were infected with 100 PFU of virus TS15-rR, a GFP-tagged epithelial tropic variant of HCMV strain Towne. After 24 h, the medium was replaced with medium containing antibodies 4-fold serially diluted in culture medium from initial 100 μg/ml (MAbs) or 5,000 μg/ml (HIG; Cytotect) stocks. The negative-control MAb is TRL298, a gB-specific MAb that lacks neutralizing activity. Representative micrographs were taken 14 days postinfection. The EC_{50}s shown below each lane were calculated from the GFP values. Note that for a full-length MAb, 100 μg/ml is equivalent to a 0.6 nM concentration.

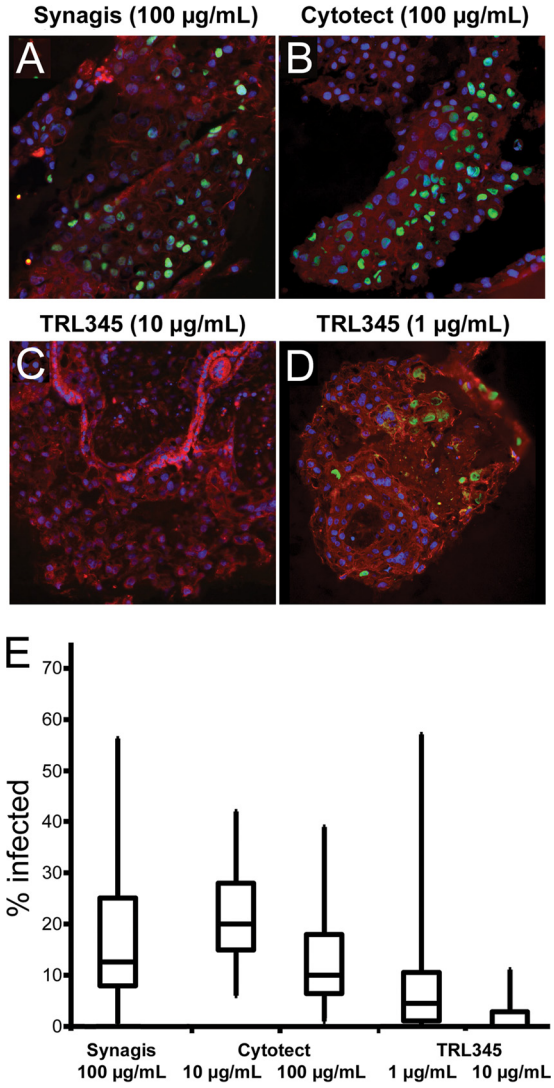

FIG 4 Infection of human chorionic villi treated with TRL345. Explants of human chorionic villi were infected with HCMV strain VR1814 preincubated with antibody. At 72 h after infection, the explants were stained for HCMV IE antigen (green), cytokeratin (red), or nuclei (blue). (A) Synagis at 100 μg/mL. (B) Cytotect at 100 μg/mL. (C and D) TRL345 at 10 μg/ml (C) and 1 μg/ml (D). (E) Percentage of cytokeratin-positive cells in trophoblast cell columns that were IE positive was determined; the central horizontal bar marks the median, with the box showing the 2nd to 3rd quartiles and the vertical lines showing the 1st to 4th quartiles. Note that for a full-length MAb, 1 μg/ml is equivalent to a 6 nM concentration.

isolates (Table 2). Although gB genotype 4 was not represented in this set, the Towne strain represents a type 4 virus, and it was also neutralized by TRL345 (Table 1).

Protection against HCMV spread within epithelial cell monolayers. HCMV persistence in spite of robust humoral responses suggests that *in vivo*, the virus may evade neutralizing antibodies by spreading from cell to cell (63). This transmission mode has been found in xenografts of human placental villi in a SCID-hu model of vascular remodeling (64, 65) and may be especially relevant in transplant-associated HCMV disease. To measure the spread inhibition, ARPE-19 epithelial cell monolayers were infected with 100 PFU of TS15-rR, a GFP-tagged epithelial-tropic variant of the Towne strain of virus. After 24 h, the culture medium was replaced with medium containing serial dilutions of HIG (Cytotect) or MAbs (TRL345 or a nonneutralizing isotype control, TRL298), and the subsequent spread of virus from the initially infected cells was monitored by GFP fluorescence (63). As shown in Fig. 3, the cultures treated with the negative-control MAb TRL298, a gB-specific MAb that lacks neutralizing activity (data not shown), became 100% GFP positive by day 14 after infection. In contrast, the cultures treated with TRL345 or with HIG (Cytotect) exhibited a dose-responsive inhibition of viral spread, and at high concentrations, viral infection was limited to small GFP-positive foci. In this assay, TRL345 was approximately 10-fold more potent than HIG.

Protection from HCMV infection in anchoring villus explants of the placenta. HCMV is entirely species specific, restricted to infecting human cells. Thus, there are no animal models that faithfully recapitulate the mechanism and kinetics of placental villus infection. To further characterize TRL345 neutralizing activity, we used an established *ex vivo* model in which explants of first trimester human placenta were grown on Matrigel (59). Within 24 h, the villus cytotrophoblasts proliferate and differentiate, forming cell columns that develop further into anchoring villi that attach the explant to the Matrigel substrate. VR1814

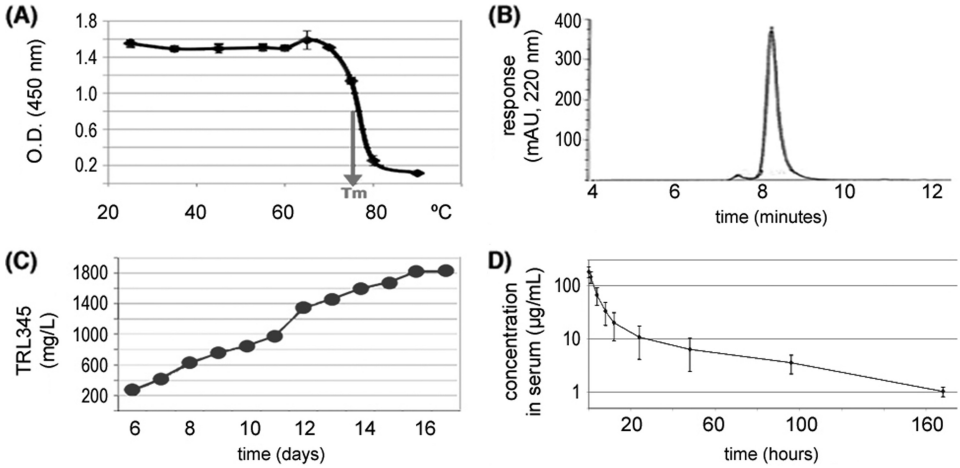

FIG 5 Stability, expression, and pharmacokinetics of TRL345. (A) TRL345 was incubated for 10 min at the indicated temperatures and then immediately applied to an ELISA plate coated with peptide antigen. Bound MAb was measured using an HRP-labeled secondary antibody, followed by HRP substrate. Tm, melting temperature; O.D., optical density. (B) Size exclusion chromatography profile of TRL345 following clarification by filtration, protein A purification, and polishing by ion exchange and hydroxyapatite chromatography. mAU, milliabsorbance units. (C) Expression level of TRL345 in stably transformed CHO cells. (D) Elimination curve of TRL345 in male Sprague-Dawley rats ($n = 3$) after a single i.v. dose at 10 mg/kg; the mean and standard deviation (SD) values are shown.

incubated with a negative-control MAb of the same isotype against respiratory syncytial virus (Synagis) readily infected the cell columns, as demonstrated by staining of the viral IE1 antigen as a marker of infection and cytokeratin as a marker for cytotrophoblasts. Incubation with HIG (Cytotect) showed a trend toward efficacy but did not reach a statistically significant effect on the median percentage of HCMV-positive cells relative to that of the control; at 100 μg/ml, the median was 12.5%, and at 10 μg/ml, the median was 19.5%. In contrast, incubation with TRL345 dramatically reduced infection at both 10 μg/ml (median, 0%) and 1 μg/ml (median, 4.4%). Accordingly, the bioequivalent dose of the monoclonal TRL345 was >50-fold lower than that of the polyclonal HIG (Fig. 4), consistent with the >50-fold higher potency observed in cell culture. The effects of Cytotect at 100 μg/ml and of TRL345 at both concentrations were all significant when the mean values were compared with that of the control antibody (Synagis), with a P value of <0.01 for Cytotect at 100 μg/ml and a P value of <0.00001 for TRL345. Further, the differences between the mean values for Cytotect at 100 μg/ml and for TRL345 at both concentrations were also significant, with P values of <0.05 (1 μg/ml) and <0.00001 (10 μg/ml). Note that for a full-length MAb, 1 μg/ml is equivalent to 6 nM concentration.

Stability, expression, and pharmacokinetics. As previously described, a total of 15 CDR residues were somatically mutated in TRL345, with 4 in the light chain and 11 in the heavy chain (44). None of these introduced new stability risks (such as deamidation, glycosylation, acidic cleavage, isomerization, or oxidation motifs) (66). The melting temperature of TRL345 was determined to be 70°C (Fig. 5A). No tendency to aggregate was observed by size exclusion HPLC (Fig. 5B). High expression (1.8 g/liter at the primary fermentor level) has been achieved in stably transformed CHO cells using the GPEx expression system (Catalent Pharma Solutions, Madison, WI) (Fig. 5C).

TRL345, administered as a single bolus i.v. injection at 10 mg/ml, was well tolerated in male Sprague-Dawley rats ($n = 3$). The pharmacokinetic parameters, comparable to those of other human MAbs administered to rats (67), are presented in Table 3, and the elimination curve is presented in Fig. 5D. The T_{max} occurred 5 min after the end of dosing, and the C_{max} was 184 μg/ml. The elimination curve, accurately described by an open two-compartment pharmacokinetic model, showed a short distribution phase, with a $t_{1/2}$ of 3 h, and a longer elimination phase, with a $t_{1/2}$ of 46 h. The volume of distribution (V) was approximately equivalent to the plasma volume (60 ml/kg); however, the volume of distribution at steady state (V_{ss}), 305 ml/kg, indicated some distribution into the extravascular space. The $AUC_{0-\infty}$ was 1,642 μg · h/ml.

Tissue cross-reactivity profile. TRL345 was applied at 1 and 20 μg/ml to a panel of cryosections of 38 adult and 21 fetal (0 to 6 months) human tissue samples of diverse origins (Charles River Laboratories, Reno, NV) (68). Various types of nonspecific staining occurred, including incompletely quenched endogenous peroxidase staining (cytoplasmic staining of a few individual cells). The assay included positive and negative controls, which were HEK293 cells transiently transfected with gB or mock-transfected cells, respectively. As summarized in Table 4, at 1 μg/ml, most specimens scored negative, with a few that showed sporadic intracellular staining in some but not all samples of a few tissue types. At 20 μg/ml, intracellular staining was seen in adult skin, colon, small intestine, and pancreas samples, as well as in fetal small intestine and pancreas samples, although again, the stained tissue

TABLE 3 Pharmacokinetic parameters for TRL345 after a single i.v. dose administered to male Sprague-Dawley rats ($n = 3$) at 10 mg/ml

Parameter	Mean	SD
C_{max} (μg/ml)	184	49
T_{max} (min)	5.1	
$t_{1/2\alpha}$ (h)	3	0.2
$t_{1/2\beta}$ (h)	46	15
V (ml/kg)	60	16
V_{ss} (ml/kg)	305	200
CL (ml/h/kg)	7	3
AUC_{0-last} (μg · h/ml)	1,584	637
$AUC_{0-\infty}$ (μg · h/ml)	1,642	631

TABLE 4 TRL345 tissue reactivity profile

Tissue	% stained cells (degree of staining), no. of donors/total donors by concn[a]: 1 μg/ml	20 μg/ml	Notes
Positive control	100 (++++)	100 (++++)	HEK293(gB)
Negative control	0 (−−)	0 (−−)	HEK293(vector)
Skin	<25 (++) cytoplasmic, 1/3	25–75 (+++) cytoplasmic, 1/3	Squamous epithelium
Colon	Negative	<25 (++) cytoplasmic, 2/3	Intestinal epithelium
Small intestine (adult)	Negative	25–50 (++) cytoplasmic, 3/3	Intestinal epithelium
Small intestine (fetal)	25–50 (++) cytoplasmic, 1/3	25–75 (+++) cytoplasmic, 3/3	Intestinal epithelium
Pancreas (adult)	25–75 (+++) cytoplasmic, 1/3	25–75 (+++) cytoplasmic, 2/3	Duct epithelium
Pancreas (fetal)	Negative	25–50 (+) cytoplasmic, 2/2	Acinar epithelium
Others[b]	Negative	Negative	

[a] Intensity of stain: **+**, light; **++**, light to moderate; **+++**, moderate; **++++**, dark.
[b] Others include adrenal, bladder, white blood cells, blood vessels (endothelium), bone marrow, breast, cerebellum, cerebral cortex, eye, fallopian tube, heart, kidney, liver, lung, lymph node, muscle (striated), nerve (peripheral), parathyroid, parotid (salivary gland), pituitary, placenta, prostate, spinal cord, spleen, stomach, testis, thymus, thyroid, tonsil, ureter, uterus (cervix and endometrium).

was not uniformly positive, and there was variability across the samples from different individuals. A few donors were overrepresented in the positively stained samples (including staining of multiple tissues), suggesting a systematic difference. The HCMV-seropositive status of the tissue donors was not available. A positive control (staining for the ubiquitous CD31 antigen) was used to qualify the tissue samples, but factors, such as age or time between death and tissue preparation, which may have contributed to the result, were also not available.

An NCBI BLAST search of the epitope on gB only identified 9 proteins with >75% homology to AD-2, and none had a reported tissue distribution corresponding to the staining pattern seen with the high concentration of TRL345. In a published study of a MAb with off-target reactivity, the pharmacokinetics was substantially abnormal (69), but no such effect was observed for TRL345, consistent with all of the sporadic staining being intracellular.

DISCUSSION

The leading small-molecule antiviral effective against HCMV, valganciclovir, has associated side effects (including neutropenia) that preclude its use for major indications, including congenital transmission and the early posttransplant period for hematopoietic stem cell transplantation (70). Antibody therapy provides an alternative with potentially lower toxicity. Studies of hyperimmune globulin (HIG) have shown indications of efficacy against HCMV in both congenital and transplant settings (71, 72). Compared to HIG, an MAb offers more uniform manufacturing with lower risk of contamination by viruses or prions, has lower potential for off-target reactivity leading to toxicity, and is simpler to administer due to the higher potency.

MSL-109, an early MAb against the gH component of the pentameric complex, was tested clinically for activity in HCMV-associated retinitis in HIV-infected patients, but no efficacy was seen (73). More recent studies have shown that HCMV rapidly develops resistance to MSL-109 *in vitro* (37).

An effective MAb treatment for HCMV infection needs to protect a wide variety of cells against infection by the full range of circulating virus strains. Several viral envelope antigens have been proposed as being critical for infection (28, 36, 37, 74–76). For example, the failure of gH/gL to be incorporated into the virion (via the deletion of gO) resulted in a failure to infect fibroblasts and endothelial and epithelial cells (77). It has been clearly established for many years that gB acts as a fusion protein essential for cell entry (78), with more recent work showing that it interacts with other proteins that regulate cell tropism, including gH (79). TRL345 exhibited equipotent neutralization of infection in fibroblast, smooth muscle, endothelial, and epithelial cells, and it prevented HCMV spread in ARPE-19 epithelial cells *in vitro*. The protection of fibroblasts was further verified for all 15 tested primary HCMV isolates of diverse genotypes.

Although multiple targets may be involved in infection, the AD-2 site I epitope on the gB envelope glycoprotein is of particular interest because it is nearly invariant and poorly immunogenic (29). Both properties are found in critical function epitopes on other viruses, including respiratory syncytial virus (RSV), hepatitis C virus (HCV), and influenza virus (80–82). Since other regions of the virus are highly polymorphic, including gH, for example, the natural history of the virus suggests that escape from TRL345 will be rare. TRL345 has a K_D of 1 pM for the AD-2 site I epitope. Its potencies for neutralizing VR1814 *in vitro* and for the protecting placental explants were 50-fold better than those of the commercially available HIG product Cytotect and 5-fold better than those of the well-known comparator anti-gB MAb ITC-88 (28). In both placental and posttransplant infections, multiple host cells play roles in pathology. Fibroblasts represent a potential reservoir of particular interest, since virus produced in fibroblasts can infect many cell types, whereas virus produced in endothelial cells shows a strong tropism for that cell type (63). Likewise, ubiquitous smooth muscle cells represent a potential site for infection leading to vascular inflammation, which is a risk factor for heart transplant patients (83). The infection of both of these cells has been documented in the blood vessels of floating villi and the chorions of congenitally infected placentas (84). An MAb with the epitope specificity of TRL345 is of particular interest in light of published clinical data on a well-defined subset of renal transplant patients. Of 14 patients who lacked detectable antibodies to gB (AD-2), 4 (29%) required antiviral therapy, whereas none of the 31 patients with a high titer required therapy ($P < 0.008$) (85).

Relevant to congenital transmission, TRL345 prevented infection in placental explants by HCMV. The neutralizing activity of TRL345 was compared to that of TRL310, which was constructed based on the published sequence of MAb 1F11, a low-pM-affinity MAb against the pentameric complex (36). Although the potency for protecting endothelial cells was <1 ng/ml, TRL310 failed to

completely protect smooth muscle cells, placental fibroblasts, and trophoblast progenitor cells from infection even at a 4-fold higher concentration (10 μg/ml = 60 nM). In contrast, TRL345 had a similar potency on all cell types tested, ~100 ng/ml (0.6 nM).

TRL345 is resistant to thermal denaturation or formation of aggregates and has been expressed in stably transformed CHO cells at a level suitable for commercial-scale production. Human MAbs as a group have excellent safety properties (86). In tissue reactivity profiling, only sporadic staining was observed, all of which was intracellular and thus unlikely to be relevant to clinical use in which the MAb will be administered systemically. Freezing, cutting, and fixation of the tissues disrupt cells and expose intracellular epitopes that are not normally accessible *in vivo* and that may be denatured during processing of the tissue; positive staining of some or many tissues that do not correlate with any known epitope or any *in vivo* effects are common (68). Consistent with the absence of binding to normal tissues, normal pharmacokinetics was observed (69). TRL345 administered to rats as a single i.v. dose (10 mg/kg) was well tolerated, and the pharmacokinetics profile was well described by an open two-compartment model with a mean elimination half-life of 46 ± 8 h.

Based on the properties described here, including expression on a commercial scale, TRL345 is being prepared for a clinical evaluation of its efficacy in reducing HCMV infection in both transplant and congenital transmission settings.

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We honor the memory of project leader and friend Bill Usinger.

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